The roles and regulation of the *Drosophila* Lgl tumour suppressor in cell division

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Declaration

I, Graham P. Bell, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Cell polarity is a fundamental feature of most cells, and is required for a diverse range of cell functions. A conserved set of proteins is involved in the establishment and maintenance of cell polarity. Several of these polarity proteins in *Drosophila* are recognized as neoplastic tumour suppressors, and loss of cell polarity in general has been linked to cancer malignancy. Although much is known about the general role of the polarity proteins, many questions still remain about the details and extent of their functions. I have focused on the Lgl protein in *Drosophila* epithelia: a basolaterally-localized tumour suppressor. In mitosis, Lgl undergoes a dramatic relocalisation away from the plasma membrane into the cytoplasm. I have found that this is regulated by direct phosphorylation by the Aurora cell cycle kinases: this is in contrast to the regulation of Lgl in interphase polarity, which is mediated by aPKC. The mitotic relocalisation event appears to play an important role in the correct orientation of the mitotic spindle and cell division in epithelia, which may be necessary for maintaining tissue integrity. Thus in addition to the established role for Lgl in cell polarity, my work suggests a novel function for Lgl during mitosis.

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Abbreviations

Vertebrate homologs of *Drosophila* proteins are given in square brackets.

(F)ERM	(4.1 protein)Ezrin/radixin/moesin
(hs)flp	(Heat-shock) Flippase
(MA)GUK	(Membrane Associated) Guanylate kinase
AFG4	Actin flip-out GAL4
APC	Adenomatous polyposis coli
aPKC	Atypical protein kinase C
Arm	Armadillo/β-catenin
ATP	Adenosine triphosphate
Aur A/B	Aurora A/Aurora B
Baz/Par-3	Bazooka/Partioning defective protein 3
BBT	PBT with BSA
Bora	Aurora borealis
BSA	Bovine serum albumin
Brat	Brain tumour protein
Cdc42	Cell division control protein 42
Cdk1	Cyclin-dependent kinase 1
CENP-A	Centromere protein A
CNN	Centrosomin
Crb	Crumbs
DAPI	4',6-diamidino-2-phenylindole
Diap1	*Drosophila* inhibitor of apoptosis 1
Dlg	Discs large
ECad	E-Cadherin
ECR1	Evolutionary conserved region 1
Ect2	Epithelial cell transforming sequence 2 oncogene)
ELAV	Embryonic lethal abnormal visual system
EMT	Epithelial-mesenchymal transition

Ey	Eyeless
Frt	Flippase recognition target
GFP	Green fluorescent protein
Gαi	Guanine nucleotide binding protein subunit α
Hh	Hedgehog
HPLC	High-performance liquid chromatography
HPV	Human papilloma virus
IKNM	Interkinetic Nuclear Migration
Insc	Inscuteable
Jup	Jupiter
Khc73	Kinesin heavy chain 73
Lgl	Lethal giant larvae
LKB1	Liver kinase B1
LRR	Leucine Rich Repeat
MARCM	Mosaic analysis with repressible cell marker
MF	Morphogenetic furrow
Mir	Miranda
MRLC	Myosin Regulatory Light Chain
Mud [NuMA]	Mushroom body defect [Nuclear mitotic apparatus protein]
Myo-II	Myosin II
Myr	Myristoylated
NGS	Normal goat serum
Nls	Nuclear localisation signal
Par-6	Partioning defective protein 6
PATJ	PALS1-associated TJ protein
PB1	Phox and Bem 1
PBS	Phosphate-buffered saline
PBT	PBS with Triton X-100
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PH3	Phosphohistone H3
Pins [LGN]	Partner of Inscuteable [Leu-Gly-Asn repeats]

Pon	Partner of Numb
Ptc	Patched
ROCK	Rho-associated protein kinase
SOC	Super-optimal broth with catabolite repression
Sqh	Spaghetti squash
Sdt [PALS1]	Stardust [Membrane-associated palmitoylated protein 5]
Taz	Transcriptional coactivator with PDZ-binding motif
TBS(T)	Tris-buffered saline (with Triton X-100)
TPR	Tetratricopeptide repeat
Tub	Tubulin
UAS	Upstream activating sequence
Yki [YAP]	Yorkie [Yes-Associated Protein]

Chapter 1. Introduction

1.1 Cell polarity

The development of individual organisms and more general process of evolution both display a shared likeness in the transition from simple, progenitor states to a beautifully diverse and widespread larger system. At its core, developmental biology can be thought to pose one general question: how is it that a single cell can lead to the complexity of a fully grown organism, encompassing manifold cell, tissue and organ types. For example, a tissue comprises a population of specialised cells resulting in a structure with a specific function; despite each cell sharing the same genetic make-up. The specialisations that tissues display can also be seen at both the cellular and subcellular level. Although numerous and highly varied cell types can arise from a single cell – discussed further in Section 1.4 – the basis and allowance of these specialisations depends upon a fundamental feature of most cells: polarity. Most animal cells display a clear cell polarity – an asymmetric organisation of different aspects of the cell, such as subcellular components and general molecular asymmetries. Cell polarity is required for a range of highly diverse processes, such as cell migration, tissue integrity, and the aforementioned specialisation of cell fates through asymmetric cell division (Etienne-Manneville, 2008; Knoblich, 2001; Noatynska and Gotta, 2012). Despite the broad importance of cell polarity, the underlying principles are highly similar across all cell types and organisms. These include the presence of dedicated polarity proteins (or 'polarity determinants'), which specify and control the asymmetries of the cell and its constituents.

Examples of some polarised cells and their roles are shown in Figure 1.1. Perhaps the best-studied polarised cells are epithelial cells. The epithelium is the first tissue type to arise in embryonic development, and comprises one of the four basic types of animal tissue - the others being connective tissue, muscle tissue and nervous tissue (Tyler, 2003). Epithelial tissues cover the open surfaces of the body, including the skin, and line the cavities of the body. This Introduction and the work that follows focus largely on epithelial cells as a model, but it is important to note that cell polarity itself, and the regulation thereof, are more fundamental and apply

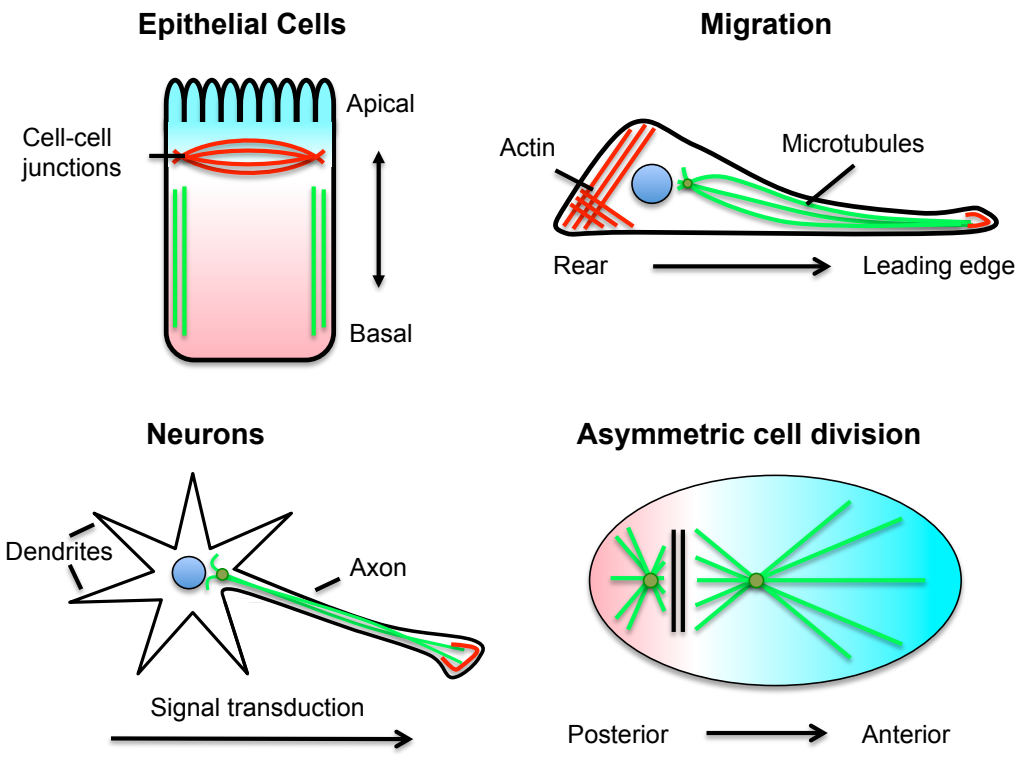

Figure 1.1 Polarised cell types

Polarity is a fundamental feature of most cells and required for diverse processes like migration, signalling, and asymmetric division. Adapted from www.eb.tuebingen.mpg.de/typo3/temp/pics/c3cb786a9c.jpg

to most cell types. A cartoon of a polarised epithelial cell is shown in Figure 1.1 (top left). Cell polarity results in asymmetric distribution of components and allows specialisations. For example, the apical region of the cell faces the external environment or a lumen. Typical specialisations include microvilli, for the absorption of extracellular material, and receptors or secretory channels to respond to and/or send signals. A sub-apical region is designated in some cells, lying just basal to the apical region proper, but still determined by the apical polarity proteins.

Basal to the sub-apical region lies an adhesive belt, comprising the adherens junctions, which form the mechanical connections between neighbouring cells. Tight junctions – or septate junctions in *Drosophila* – act as paracellular diffusion barriers between cells and are localised laterally. The basal surface of epithelial

cells contacts the extracellular matrix, and features hemidesmosomes (Harris and Tepass, 2010; Knust and Bossinger, 2002; Tepass et al., 2001).

A single polarised cell, through the asymmetry of its molecular components, can therefore be specialised to perform a variety of functions. An aggregation of multiple polarised cells together similarly leads to a polarised and functional tissue. The polarity of cells and tissues is important for an organised and functional system, but loss of polarity is also implicated in cancer development, elaborated in 1.5, but briefly shown here (Ellenbroek et al., 2012; Macara and McCaffrey, 2013; Wodarz and Nathke, 2007). The general organisation and structure of a tissue, reliant on the cell-cell adherens junctions, and the normal function of a cell/tissue, is disrupted if cells lose polarity. The loss of cell polarity, cell shape and disrupted tissue architecture contributes to an amorphous mass of cells, rather than an organised layer. Particularly, the loss of cell polarity may assist in cells escaping from the ordered and regulated epithelial layer to form metastases (Macara and McCaffrey, 2013). Further details are given later.

1.2 *Drosophila* as a model to study cell polarity

Epithelial polarity is dependent on the actions of a number of dedicated polarity proteins (or, polarity determinants). Much of the research on epithelial polarity has been carried out in *Drosophila* and *C. elegans*, although the mechanisms are remarkably similar in vertebrates (see (St Johnston and Ahringer, 2010; Tepass, 2012) and (Chen and Zhang, 2013) for comprehensive reviews). One main difference between these model organisms and vertebrates is the position of the lateral cell junctions (Knust and Bossinger, 2002). In vertebrates, tight junctions are positioned apically to the adherens junctions and act as paracellular diffusion barriers. In *Drosophila*, it is the septate junctions that perform this role, but they are positioned basally to the adherens junctions (in most tissues – in some gut cells the structure is more similar to vertebrates, with septate junctions apical to adherens junctions (Baumann, 2001)). *C. elegans* junctions more closely resemble the *Drosophila* arrangement.

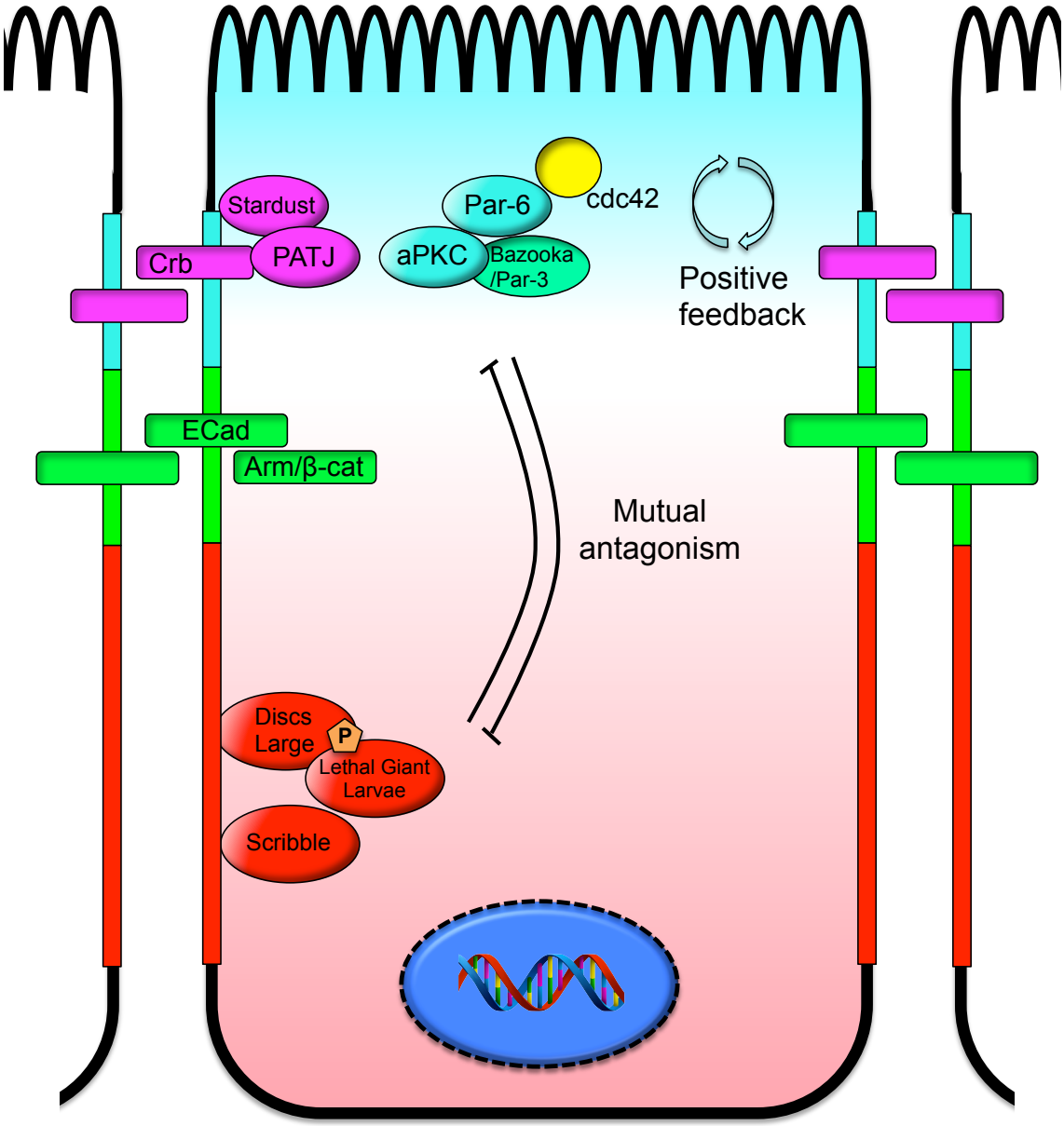

Figure 1.2 Epithelial Polarity in *Drosophila*

Polarity in *Drosophila* epithelia is characterised and determined by three main complexes: the apical Crumbs (magenta) and aPKC/Par-6 (cyan) complexes, and the basolateral Scribble module (red). Interactions within and between these complexes establish and maintain polarity: most notably mutual antagonism between apical and basal complexes. Most polarity determinants are conserved from *Drosophila* to vertebrates (see text for details). Adherens junctions (green) mechanically link neighbouring cells together.

The key polarity proteins in epithelial cells are broadly considered to fall in one of three polarity complexes: the apical Crumbs and aPKC/Par-6/Par-3 complexes, and the basolateral Scribble complex.

1.2.1 The aPKC/Par-6/Par-3 complex

The PAR proteins were originally identified and characterised in *C.elegans*, using the one-celled embryo as a simple and amenable model to study polarity regulation and its initiation. The *C.elegans* embryo develops from a uniform state, to a highly polarised embryo, before undergoing asymmetric cell divisions leading to different cell fates. The initial steps in polarisation are not discussed here, but involve a polarity cue that causes asymmetric regulation of the actin cytoskeleton (St Johnston and Ahringer, 2010). A consequence of this is the asymmetric localisation of members of the PAR protein family, along with aPKC. The PAR proteins are named for PARtitioning Defective Proteins, since their loss results in perturbed domain size and division of the *C.elegans* embryo (Cowan and Hyman, 2007; Goehring, 2014; Gonczy, 2008; Hoege and Hyman, 2013). PAR-6 and PAR-3 are localised to the anterior pole of the embryo after polarity induction, and PAR-1 and PAR-2 to the posterior (Boyd et al., 1996; Etemad-Moghadam et al., 1995; Guo and Kemphues, 1995; Hung and Kemphues, 1999; Watts et al., 1996). Interactions between the PAR proteins help to determine domain size and embryo polarity, by phosphorylation-mediated exclusion from the domains, and unclear mutual antagonisms (Cowan and Hyman, 2007; Goehring, 2014; Goehring et al., 2011a; Goehring et al., 2011b; Gonczy, 2008; Hoege and Hyman, 2013). Similar mechanisms play important roles in *Drosophila* polarity, which will be elaborated below.

In *Drosophila* epithelia, aPKC and Par-6 are localised to the apical and sub-apical regions and are key proteins in determining this domain of cells (Petronczki and Knoblich, 2001; Rolls et al., 2003; St Johnston and Ahringer, 2010; Wodarz et al., 2000). PAR-3 in *C.elegans* localises with aPKC and PAR-6 (Gonczy, 2008; Hoege and Hyman, 2013; Noatynska and Gotta, 2012), but in *Drosophila* Par-3/Bazooka is also found at the level of the adherens junctions (Harris and Peifer, 2005; Morais-de-Sa et al., 2010). Although Baz can bind to aPKC, it is also a target of aPKC

phosphorylation, which results in the removal of Baz from the apical complex (Izumi et al., 1998; Morais-de-Sa et al., 2010; Wirtz-Peitz et al., 2008). Baz is also phosphorylated by Par-1 to exclude it from the lateral domain (Benton and St Johnston, 2003b), by interfering with Baz oligomerisation, which is necessary for membrane association (Benton and St Johnston, 2003a). Both *C.elegans* PAR-3 and the *Drosophila* ortholog act in the initial formation of adherens junctions, and may assist in junctional maintenance (Chen and Macara, 2005; Harris and Peifer, 2004; Knust and Bossinger, 2002).

aPKC, Par-6 and Baz were each reported as being able to bind the others *in vitro* (Betschinger et al., 2003; Joberty et al., 2000; Lin et al., 2000; Noda et al., 2003; Suzuki et al., 2001; Tabuse et al., 1998; Wodarz et al., 2000)(and see Figure 1.3) and aPKC and Par-6 are important in determining the apical domain: either loss or over-expression can affect cell polarity (Lee et al., 2006b; Rolls et al., 2003; Tepass, 2012). Par-6 is also able to interact with members of the apical Crumbs complex, see below and (Tepass, 2012), whilst aPKC phosphorylates Lgl, a member of the Scribble module (Betschinger et al., 2003; St Johnston and Ahringer, 2010). It has been suggested that Par-6 inhibits aPKC activity (Atwood et al., 2007; Wirtz-Peitz et al., 2008; Yamanaka et al., 2001), or activates it (Graybill et al., 2012): but either way somehow affecting the kinase activity of aPKC. Consistent with its ability to activate aPKC, loss of Par-6 causes similar defects to *aPKC* mutants (Petronczki and Knoblich, 2001; Wodarz et al., 2000). aPKC is also able to phosphorylate Crumbs (Sotillos et al., 2004), and this might stabilise Crumbs at the apical membrane (Fletcher et al., 2012). Interestingly, a recent study found that kinase-dead alleles of aPKC, or a mutant unable to bind Par-6, did not show the dramatic loss-of-polarity phenotype that the commonly–used *aPKC null* mutant allele does (Kim et al., 2009). Rather than the kinase activity of aPKC being critical for cell polarity, with Par-6 and Baz acting primarily as scaffolds to localise aPKC correctly, as previously suggested e.g. (Lin et al., 2000), the authors posit some other unknown functions of aPKC (Kim et al., 2009). However, the suggestion that aPKC kinase activity is not important for the polarisation of follicular epithelium (Kim et al., 2009) contrasts with other evidence that supports a role for the phosphorylation of Bazooka by aPKC as a key step in this process (Morais-de-Sa et al., 2010). Nevertheless, the numerous interactions between apical polarity determinants, and

the potential importance of feedback mechanisms between them also (Fletcher et al., 2012), suggest a more complicated system than previously thought.

1.2.2 The Crumbs complex

The Crumbs complex is a second apical complex, consisting of the transmembrane protein Crumbs, the MAGUK protein Stardust (PALS1 in vertebrates), PATJ and Lin7 (Tepass, 2012; Tepass and Knust, 1993; Tepass et al., 2001; Tepass et al., 1990). The short cytoplasmic tail of Crumbs contains a FERM-binding domain, by which it associates with β-spectrin and Yurt (Laprise et al., 2006; Medina et al., 2002; Tepass, 2012); and an ERLI motif, which mediates interactions with Stardust and Par-6 (Bachmann et al., 2008; Bachmann et al., 2001; Hong et al., 2001; Lemmers et al., 2004). Stardust is able to bind to Par-6 via ECR1 regions, and to PATJ via L27 domains (Bulgakova and Knust, 2009; Tepass, 2012). Similarly to the Par-6 complex, members of the Crumbs complex are important for specification of the apical domain (Hong et al., 2003; Tepass and Knust, 1993; Wodarz et al., 1993; Wodarz et al., 1995), although Crumbs is absent from some polarised cell types, such as neuroblasts and adult mid-gut intestinal epithelium (Tepass, 2012). Loss or over-expression of Crumbs – or portions of it – lead to a loss or over-expansion of the apical domain respectively (Tepass, 2012; Wodarz et al., 1993; Wodarz et al., 1995).

Despite the aPKC/Par-6/Par-3 and Crumbs complexes usually being defined as two complexes, there are numerous interactions between them. The interaction between Crumbs and Par-6 is thought to prevent the binding of Par-6 to Baz (Morais-de-Sa et al., 2010; Walther and Pichaud, 2010). Par-6 is also able to bind to Stardust, and aPKC interacts with PATJ (Hurd et al., 2003; Tepass, 2012; Wang et al., 2004). Phosphorylation of Crumbs by aPKC, and the formation of Crumbs homodimers between neighbouring cells, helps stabilise Crumbs at the apical membrane (Fletcher et al., 2012). Par-6 also binds to Cdc42, another apical polarity protein (Tepass, 2012). The network of interactions between these proteins hints at a more complex picture than previously thought, and new roles for some polarity proteins have recently been posited. In neuroblasts, Baz is responsible for recruiting aPKC/Par-6 to the apical membrane, and aids in specifying aPKC kinase

activity (Wirtz-Peitz et al., 2008); whereas in epithelia Baz is excluded from the apical domain and aids in junction formation and positioning (Morais-de-Sa et al., 2010). Similarly Crumbs is present in most epithelia, but not other polarised cell types like neuroblasts (Tepass, 2012). PATJ has also been suggested as only acting as a supporting player in apico-basal polarity, with the importance of this role increasing in more complicated and tightly regulated systems like photoreceptors (Penalva and Mirouse, 2012; Sen et al., 2012; Zhou and Hong, 2012).

1.2.3 The Scribble module

The third key group of polarity proteins, the Scribble module, comprises Scribble, Discs Large (Dlg) and Lethal Giant Larvae (Lgl), and localises laterally. Dlg and Scribble are enriched at the septate junctions, whilst Lgl is localised all around the basolateral cell membrane. These three proteins are referred to as neoplastic tumour suppressors, since the loss of any one of them results in striking over-proliferation and loss of polarity (as their names suggest) (Bilder, 2004; Bilder et al., 2000). Despite their (interdependent) co-localisation and almost identical phenotypes, until recently no direct interactions had been shown between any of the three proteins - hence the term ‘module’ rather than ‘complex’. Recent work has revealed that Dlg and Lgl are able to directly bind once Lgl has been phosphorylated on a particular motif (Zhu et al., 2014). This is explored in greater detail below.

Mutation in any of the three Scribble module genes results in dramatic phenotypes in *Drosophila*. In mammalian cells, RNAi silencing results in milder phenotypes and their roles are not so clear. This may partly be due to redundancies, since mammals have two Lgl and four Dlg homologs (Elsum et al., 2012). Additionally, Dlg and Scribble perform functions in processes other than apicobasal polarity, such as planar cell polarity and cell migration (Elsum et al., 2012; Etienne-Manneville, 2009; Humbert et al., 2008; Roberts et al., 2012). Both Dlg and Scribble have also recently been described as having roles in mitotic spindle orientation (Bergstralh et al., 2013b; Nakajima et al., 2013). Dissecting functions of polarity proteins other than in polarity itself is made difficult by the dramatic

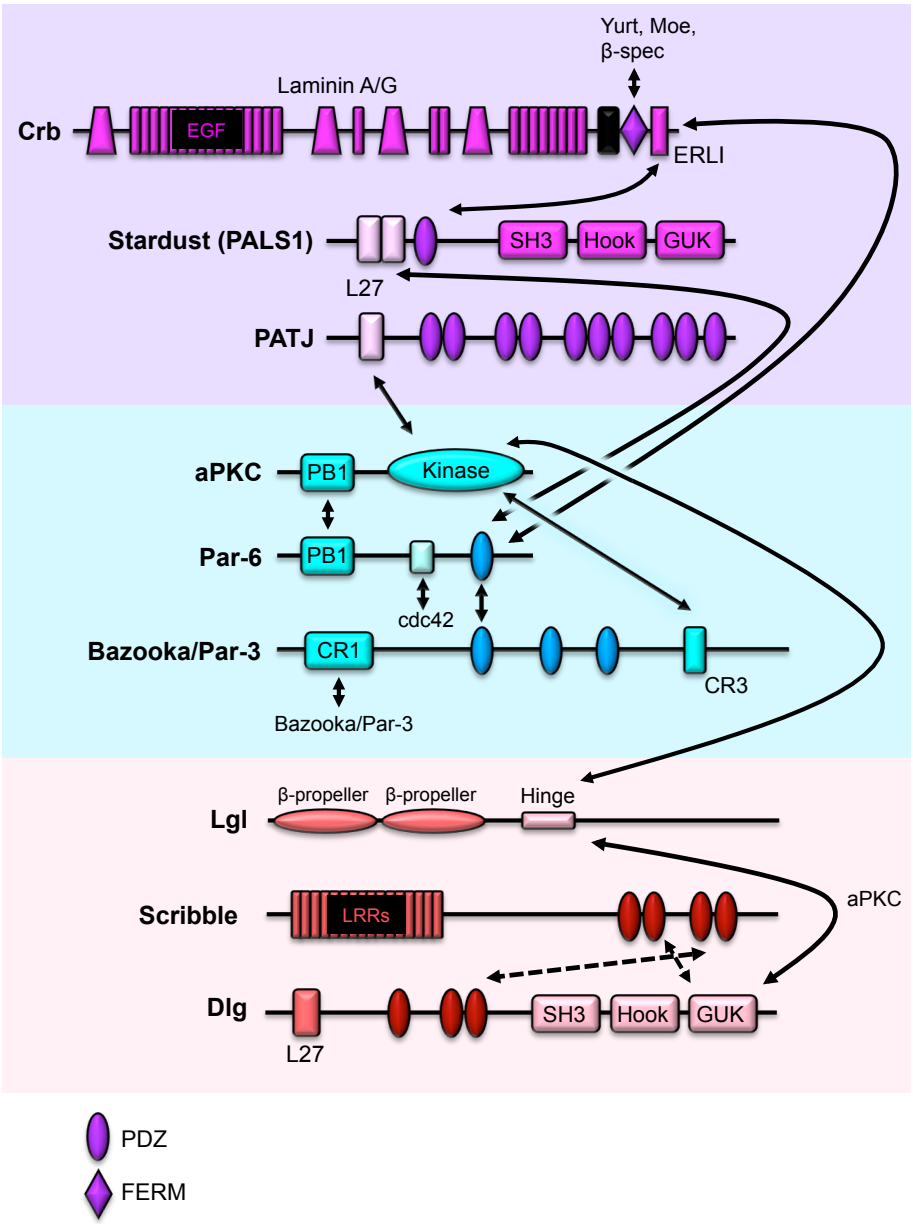

Figure 1.3 Interactions between the main polarity complexes

Interactions between polarity determinants are important for the establishment and maintenance of cell polarity, and for other functions. See text for details. Adapted from (St Johnston and Ahringer, 2010).

disruption of tissue architecture and organisation in many of the phenotypes. Work on Dlg utilised a mutant able to separate these functions: an allele of *dlg* able to rescue polarity, but missing (a portion of) the GUK domain (which is required to bind to Pins, a known player in spindle orientation)(Bergstralh et al., 2013b). Work on Scribble used RNAi to examine tissues before loss of polarity took place (Nakajima et al., 2013). This work in part attempts to elucidate a novel function for Lgl.

1.2.4 Interactions between polarity complexes: mutual antagonism

The key feature of polarity maintenance in epithelia is the mutual antagonism between the apical and basolateral polarity complexes (St Johnston and Ahringer, 2010; Tanentzapf and Tepass, 2003; Tepass, 2012). This antagonism is also present in simpler systems, such as the *C.elegans* one-celled embryo, where they help to define domain size (Hoege and Hyman, 2013) and the *Drosophila* oocyte. In epithelia, due to the additional presence of the Crumbs complex, the adherens junctions, and the slightly different role for Baz, interactions are a little more complex (St Johnston and Ahringer, 2010). Due to the interactions between the two apical complexes, it may be practical to consider them as a single complex for the purposes of polarity maintenance. Mutations of members of the Scribble module lead to a spreading of the apical domain and overall loss of polarity (Bilder, 2004; Bilder et al., 2000). This phenotype is mimicked by the over-expression of Crumbs or cdc42, which drive apical expansion (Wodarz et al., 1993; Wodarz et al., 1995) Satisfyingly, in basolateral mutants like *lgl* where the apical domain expands, the reduction of apical polarity proteins like aPKC can largely suppress the polarity phenotype (Rolls et al., 2003; St Johnston and Ahringer, 2010). Reciprocally, mutations in apical polarity proteins lead to an expansion of the basolateral domain (Petronczki and Knoblich, 2001; Rolls et al., 2003; Wodarz et al., 2000). This effect can also be partially rescued by concomitant reduction in levels of basolateral polarity proteins: mutations in members of the Scribble module can partially rescue the phenotype of *crumbs* or *stardust* mutants (Bilder et al., 2003; Laprise et al., 2009; Tanentzapf and Tepass, 2003).

Despite much work in the field, the molecular details of the antagonisms are not well understood. The consideration of aPKC as a semi-member of the Crumbs complex reveals how the Crumbs complex is able to antagonise the Scribble module. aPKC is able to phosphorylate Lgl on three conserved Serine residues – S656, S660 and S664 in *Drosophila* - and phosphorylation excludes Lgl from the apical membrane (Betschinger et al., 2003; Hutterer et al., 2004; Plant et al., 2003). It is proposed that the N-terminus of Lgl is associated with the membrane, and phosphorylation of a flexible 'hinge' region promotes a conformational change. The C-terminus of Lgl then interacts with the N-terminus, interfering with membrane association (Betschinger et al., 2005). Recently, it was shown that phosphorylated Lgl binds directly to the GUK domain of Dlg at the lateral membrane (Zhu et al., 2014). Thus the activity of aPKC results in Lgl being removed from the apical region, and also permits binding to Dlg at the lateral domain.

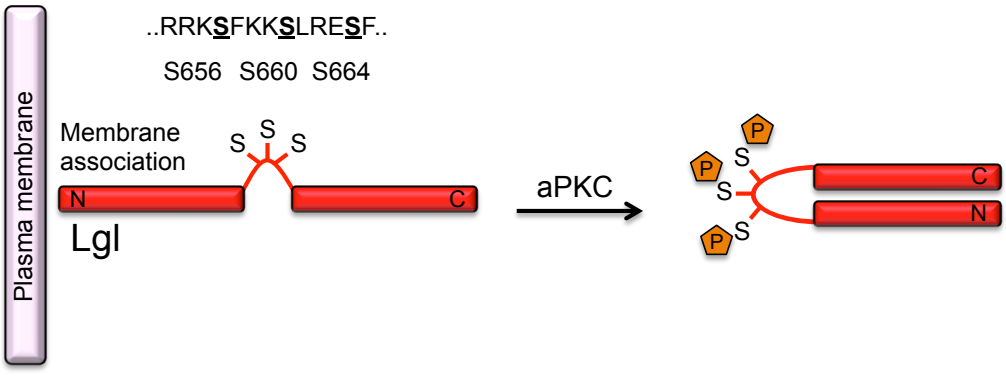

Figure 1.4 Model of Phosphorylation-mediated localisation of Lgl

aPKC phosphorylates the basolateral polarity protein Lgl to restrict it from the apical membrane. Phosphorylation on three conserved Serine residues, S656, S660 and S664 are thought to promote a conformational change and result in Lgl's disassociation from the membrane. In this thesis, the regulation and relocalisation of Lgl is revisited and we present an updated model.

How the Scribble module in turn affects the Crumbs complex is less clear. Lgl is able to associate with the aPKC/Par-6/Par-3, and may inhibit the complex's activity or localisation (Wirtz-Peitz and Knoblich, 2006; Yamanaka et al., 2003). In neuroblasts, Lgl is proposed as a 'molecular buffer' to aPKC kinase activity (Wirtz-Peitz et al., 2008). Lgl exists in a complex with aPKC and Par-6 until it is phosphorylated and excluded, allowing Baz to join the complex instead. Baz then alters aPKC substrate specificity for asymmetric cell division. The phosphorylation and exclusion of Lgl is therefore an additional layer of regulation of aPKC activity and correct cell polarity prior to division (Wirtz-Peitz et al., 2008).

A second group of basolateral polarity proteins consisting of Yurt, Coracle, the Na^+/K^+/ATPase and Neurexin IV was identified recently (Laprise et al., 2009). This complex also behaves antagonistically towards the Crumbs complex: similar to the Scribble module, loss of members of the Yurt complex leads to an expansion of the apical domain dependent on Crumbs (Laprise et al., 2009). Interestingly, in some stages of development, the Yurt group and Scribble module – singly or in conjunction - are dispensable for epithelial polarity (Laprise et al., 2009; Laprise and Tepass, 2011; Tanentzapf and Tepass, 2003), suggesting the existence of another basolateral polarity group.

1.3 Mitosis, cell shape and cell polarity

1.3.1 The Cell Cycle

The cell cycle encompasses cell growth and division, and is classified into four stages: Gap 1 (G1), synthesis (S), Gap 2 (G2) and mitosis (M). G1 and G2 primarily involve cell growth and preparation for division, S-phase involves chromosome duplication, and Mitosis is the process of cell division, where two sets of chromosomes and other cell components are segregated to opposite sites of a cell, which then divides (cytokinesis) to produce two genetically identical daughter cells. The process of cell division requires tight spatial and temporal control. Chromosomes must be correctly attached to the mitotic spindle before segregation, and the cytokinetic furrow must be aligned with the axis of chromosome segregation. In asymmetrically dividing cells, the regulation of polarity through cell division is important for cell fate and lineage specification

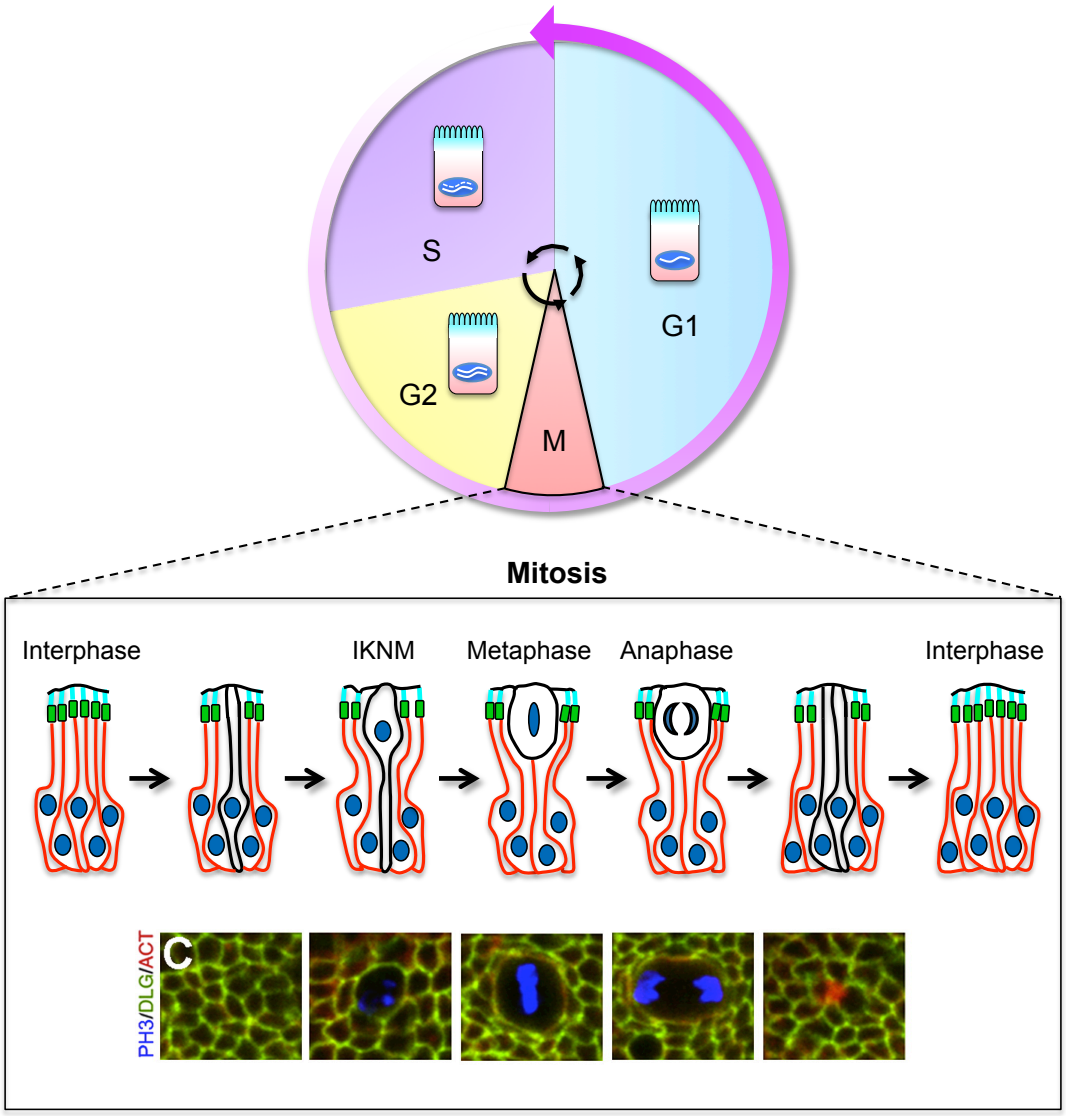

Figure 1.5 The Cell Cycle and Mitotic Phenomena

Stages of the cell cycle (top). Mitotic cells undergo cell rounding and interkinetic nuclear migration prior to cell division. Bottom panels show a top-down view of mitotic cells in a *Drosophila* wing disc (from (Meyer et al., 2011)).

(Knoblich, 2010; Siller and Doe, 2009). However, in symmetric division too, the interplay between cell shape, polarity and mitosis must also be tightly controlled, and in multicellular organisms, the local tissue architecture also depends on a carefully controlled cell division process. In contrast to temporal regulation of cell division, mechanisms controlling cell shape and polarity in mitosis are still relatively unclear.

The cytoskeleton undergoes profound remodelling in mitosis, and is important in a number of steps such as nuclear envelope breakdown, mitotic cell rounding, spindle assembly, and cytokinesis (Cadart et al., 2014; Lancaster and Baum, 2014; Thery and Bornens, 2008). Spindle formation, cytokinesis and the temporal control of cell division have been intensely studied, but mechanisms and consequences of cell shape changes – mitotic cell rounding – are still fairly undeveloped, despite having been recognised as a common feature of mitosis for a long time (Strangeways, 1922). A second phenomenon of mitotic epithelial cells is interkinetic nuclear migration (IKNM), whereby mitotic cells migrate to the apical surface of an epithelium to divide (Meyer et al., 2011; Spear and Erickson, 2012). These processes and their relationship to cell polarity are described below.

1.3.2 Mitotic Cell Rounding

At the beginning of mitosis, cells undergo profound shape changes and adopt a spherical shape for cell division, a process observed in many cell types both *in vitro* and *in vivo* (Cadart et al., 2014; Strangeways, 1922). These changes may be important for spatial requirements of cell division, including spindle assembly and orientation (Dumont and Mitchison, 2009a; Dumont and Mitchison, 2009b; Kunda and Baum, 2009; Tse et al., 2012). Major changes occur to the actin cytoskeleton, which is rearranged by action of RhoA, Myosin II and Ect2 (Maddox and Burridge, 2003; Matthews et al., 2012). Cell rounding partially depends on Rho and its effecter Rho-kinase, which phosphorylates Myosin II (Maddox and Burridge, 2003). Myosin II then accumulates around the cortex of the cell.

Disrupting actin integrity and correct localisation interferes with mitotic spindle positioning (Kunda et al., 2008; Luxenburg et al., 2011; Thery and Bornens, 2008; Thery et al., 2005). Depletion of ROCK does not fully prevent cell rounding, and in

Drosophila, the ERM protein Moesin is also important (Carreno et al., 2008; Kunda et al., 2008). Moesin is required to stiffen the cell cortex and assist in cell rounding, and depletion of Moesin affects not only cell rounding but also spindle orientation (Carreno et al., 2008; Kunda et al., 2008).

In addition to cortical enrichment of Myosin II, Moesin and actin to provide stiffness, and the disengagement of cells from their substrate (Dao et al., 2009; Harris, 1973), cells increase their intracellular pressure, acting as a balance to the cortical tension provided by the cytoskeleton (Stewart et al., 2011). Affecting this hydrostatic pressure by altering osmotic potential affects cell rounding and mitotic cell size (Stewart et al., 2011). A recent study affected cell rounding by altering the substrate stiffness and physically constraining the extent of cell rounding(Lancaster et al., 2013). Cell rounding is required for efficient progression through mitosis, effective capture of chromosomes, and stability and effectiveness of bipolar spindles (Lancaster and Baum, 2014; Lancaster et al., 2013). Although the importance of rounding is clear, how it actually affects spindle integrity is still not clear, though it may be to provide a geometrically amenable system for the reach of the microtubules (Lancaster and Baum, 2014; Picone et al., 2010; Varga et al., 2009).

Studies in cell culture provide insights in the mechanism of cell rounding. Mitotic cells within an epithelium additionally have interactions with their neighbouring cells, and shape changes cause a local deformation of the surrounding cells. The relationship with neighbouring cells, including the influence of tension that dividing cells exert and receive is an increasing area of study, and seems to affect cell division orientation and mechanics of division processes (Founounou et al., 2013; Guillot and Lecuit, 2013; Herszterg et al., 2013; Mao et al., 2011). Mitotic cell rounding is thus a common feature of cell division that involves a dramatic shape change, and impacts on the local tissue structure.

1.3.3 Interkinetic Nuclear Migration

A second common observation of mitotic cells is that they divide at the apical side of an epithelium, whereas interphase nuclei are distributed throughout the

epithelium. The apical localisation of mitotic nuclei is due to a migration towards the apical surface for cell division, before returning basally after cytokinesis: interkinetic nuclear migration. The observation that mitotic cells may be located at a different region to interphase cells was noted in the nineteenth century (Schaper, 2010), and the term IKNM was coined by FC Sauer in 1935 (Sauer, 1935); direct observation by time-lapse microscopy was observed in 2001 (Miyata et al., 2001; Noctor et al., 2001). Although the most dramatic examples of IKNM tend to occur in neuroepithelia, where the nuclei may translocate up to 100μm (Miyata, 2008), IKNM is a broadly conserved phenomena, occurring in a range of tissues and organisms (Meyer et al., 2011).

Two mechanisms have been proposed for the apical step of IKNM: migration along microtubules, and acto-myosin based movement. In many systems, treatment with microtubule or dynein inhibitors prevents efficient IKNM (Cappello et al., 2011; Gambello et al., 2003; Tsai et al., 2005). Conversely, in *Drosophila* wing discs, microtubule-inhibition affected spindle function, but not IKNM or cell rounding (Meyer et al., 2011). Affecting levels of actin or myosin did however affect apical IKNM (Meyer et al., 2011). Microtubule depolymerisation also does not affect apical IKNM in the zebrafish retina (Norden et al., 2009). The contribution of each of these mechanisms thus seems to vary depending on the tissue. The extent of IKNM varies greatly depending on the cell type, and it is proposed that the roles for microtubule- or actin- based migration depend on the cell (Spear and Erickson, 2012).

Despite observations of IKNM stretching to over a century, and a reasonable understanding of the proximal causes, the overall function of IKNM is still mysterious. An intuitive reason would be to accommodate a greater number of cells and to permit the cell shape changes that occur during mitosis. In thin cells where the nucleus results in a ‘bulge’, like neuroepithelia or *Drosophila* wing disc epithelia, pseudostratification allows more cells to be packed into a particular area than if the nuclei were all positioned at the same level. In the absence of IKNM, cells might eventually lose the pseudostratification (Spear and Erickson, 2012). It has already been described that cells undergo dramatic morphological shape changes during mitosis. By sequestering the mitotic nuclei to the apical surface, it may be more

feasible for these shape changes to occur, and permit normal cell division, away from the compact and crowded basal area where most nuclei are located. On the other hand, cell junctions are located towards the apical surface, so despite the absence of nuclei-crowding, the apical surface might be a more restrictive area to attempt dramatic shape changes.

The presence of the junctions points to another potential reason: the majority of the polarity determinants are localised towards the apical side of the cell. Even basolateral polarity proteins Dlg and Scribble are enriched at septate junctions, which are located apical to the majority of cell nuclei. Division at the apical surface might allow easier inheritance of polarity proteins and adherens junctions for maintenance of epithelial polarity and cell architecture. A final reason might be the direct role for proteins located near the apical surface in cell division. It has previously been suggested that the adherens junctions component β-catenin/armadillo and its binding partner APC2 are important for the tethering of the mitotic spindle to the cortex (McCartney et al., 2001). More recent work suggested roles for aPKC, Scribble and Dlg in mitotic spindle orientation (Bergstralh et al., 2013a; Bergstralh et al., 2013b; Guilgur et al., 2012; Nakajima et al., 2013): this is discussed in detail in the next section. Data identifying Dlg as a player in spindle orientation is well supported, and it may be that the nucleus needs to migrate to where Dlg is localised – enriched at the septate junctions, just basal to the adherens junctions – for efficient spindle orientation. The potential roles of aPKC and Scribble are less clear in this respect (see below).

IKNM and cell rounding are common mitotic phenomena, and appear to be important for correct cell division. In particular, the correct orientation and assembly of the mitotic spindle is crucial in mitosis, to ensure accurate chromosome segregation. Failure of normal chromosome segregation can lead to aneuploidy and cell death, and contributes to cancer formation. The regulation of the mitotic spindle is thus a critical aspect of cell division, and is discussed below.

1.4 Symmetric and asymmetric cell division

Multicellular organisms have a variety of different cell types, yet all develop from a single cell. In order to generate this diversity, some cells are able to produce two distinct daughter cells after cell division, in a process of asymmetric cell division. The process requires that a cell establishes a polarity axis, leading to the asymmetric localisation of cell fate determinants; and that the mitotic spindle aligns with this polarity axis, resulting in the segregation of fate determinants unequally into the daughter cells. Most cells divide symmetrically: producing two similar daughter cells. In this case, the spindle aligns perpendicular to the apico-basal polarity axis. It has been proposed that spindle misorientation could lead to cells 'escaping' the epithelium by dividing out of the epithelial layer, and that this could contribute to the progression of tumour formation, and/or metastatic potential. Many of the players and mechanisms involved in orienting the spindle are the same for asymmetric or symmetric division, though asymmetric division has attracted a greater share of interest and will be discussed first.

1.4.1 Asymmetric Cell Division and Spindle orientation

Most of our understanding of asymmetric cell division in *Drosophila* comes from work on the neuroblasts. In embryogenesis, neuroblasts delaminate from the neuroectoderm and undergo rounds of asymmetric cell division to generate neurons for the larval nervous system. After a period of quiescence from the end of embryogenesis, they again begin to undergo asymmetric division during larval development, generating the neurons of the adult fly brain (Sousa-Nunes et al., 2010). Most neuroblasts are type I neuroblasts, which divide to produce another neuroblast, and a Ganglion Mother Cell (GMC). This GMC then divides once more to generate two neurons. Some Type II neuroblasts are also present in the larval brain: these divide to give rise to another neuroblast and an Intermediate Precursor Neuron. This IPN then divides to produce another IPN, and a GMC (Boone and Doe, 2008; Sousa-Nunes et al., 2010). Not only is cell fate determined by asymmetric cell division, but also the size of the daughter cells is unequal. This is due to asymmetric positioning of the spindle, but is not dealt with here.

1.4.1.1 Neuroblast polarity

Neuroblasts become polarised during mitosis, with the accumulation of aPKC, Par-6 and Par-3 at the apical cell cortex (Morin and Bellaiche, 2011; Petronczki and Knoblich, 2001; Rolls et al., 2003; Schober et al., 1999; Wodarz et al., 2000; Wodarz et al., 1999). Inscuteable is a non-epithelial protein, which also localises to the apical cortex and is important for asymmetric division (Kraut and Campos-Ortega, 1996; Kraut et al., 1996). Inscuteable (Insc) binds Par-3 at the apical cortex, and then binds the TPR region of Pins (Partner of Inscuteable) to recruits Pins apically: thus facilitating the binding of Pins to cortical Gαi proteins (Schaefer et al., 2001; Schaefer et al., 2000; Yu et al., 2000). Pins then relinquishes Insc and instead binds to Mud, a dynein-binding protein, which links microtubules to the apical cortex to anchor the spindle and ensure division will occur in the axis of apico-basal polarity (Bowman et al., 2006; Izumi et al., 2006; Siller et al., 2006).

Cell fate determinants localise at the basal cortex: including the proteins Numb and Brat, whose localisations are facilitated by binding to Pon (Partner of Numb) and Miranda respectively (Betschinger et al., 2006; Lee et al., 2006c; Lu et al., 1998; Wang et al., 2007). Once the spindle has been aligned with apico-basal polarity, upon cell division the basal daughter cell inherits cell fate determinants Numb and Brat. Numb was the first asymmetrically localising cell fate determinant to be characterised (Rhyu et al., 1994; Spana et al., 1995). Numb is a repressor of Notch signalling, and unequal amounts of Numb in each of the daughter cells therefore results in unequal levels of Notch activity (Rhyu et al., 1994; Spana et al., 1995; Wirtz-Peitz et al., 2008). This in turn results in the positive regulation of the transcription factor Tramtrack in Notch-positive cells, and the establishment of distinct cell fates for each daughter cell (Guo et al., 1996).

Initially, it was thought that actomyosin processes moved fate determinants along the cell cortex to result in asymmetric localisation (Knoblich, 2010). Recent work has instead shown a simpler explanation. aPKC phosphorylates Numb to prevent membrane association, in a manner reminiscent of Lgl apical exclusion in epithelia (Betschinger et al., 2005; Betschinger et al., 2003; Wirtz-Peitz et al., 2008). aPKC is also able to phosphorylate the adaptor protein Miranda and exclude it similarly

(Atwood and Prehoda, 2009; Wirtz-Peitz et al., 2008). It is known that the basolateral polarity protein Lgl can affect the localisation of basal, but not apical, determinants in neuroblasts (Peng et al., 2000): this was thought to be tied in with the actomyosin role in fate determinant localisation through Lgl's binding to non-muscle myosin (Peng et al., 2000; Strand et al., 1994a; Strand et al., 1994b). Recently, a new model was proposed whereby the role of Lgl is to regulate the activity and substrate specificity of aPKC, thus ultimately affecting cell fate determinant localisation (Wirtz-Peitz et al., 2008). In this model, aPKC is initially in a complex with Par-6 and Lgl: where Par-6 inhibits aPKC activity. Lgl and Bazooka competitively bind to Par-6/aPKC, but in interphase Lgl is the preferred partner. In mitosis, the cell cycle kinase Aurora A phosphorylates Par-6 to remove its inhibition of aPKC. aPKC then phosphorylates Lgl to relocalise it to the cytoplasm. This allows Baz to join Par-6 and aPKC, where it alters aPKC substrate specificity, allowing phosphorylation of cell fate determinant Numb, and its restriction to the basal cortex (Wirtz-Peitz et al., 2008). Since phosphorylation of Numb can only occur once the pool of Lgl has been excluded by phosphorylation, Lgl is thus thought to act as a 'molecular buffer'. Non-phosphorylatable Lgl, with an 'infinite buffering capacity', or *lgl* mutants with no buffering capacity, therefore affect cell fate determinant localisation through the dysregulation of aPKC activity (Wirtz-Peitz et al., 2008). Recent work has slightly modified this model: rather than Par-6 inhibiting aPKC until phosphorylation by Aurora prevents this inhibition, Par-6 has been proposed as an activator of aPKC activity (Graybill et al., 2012). Similarly, in this work we propose that aPKC is responsible for polarising Lgl localisation, but Aurora is responsible for cytoplasmic relocalisation. Nonetheless, this model of polarity in neuroblasts remains largely accurate.

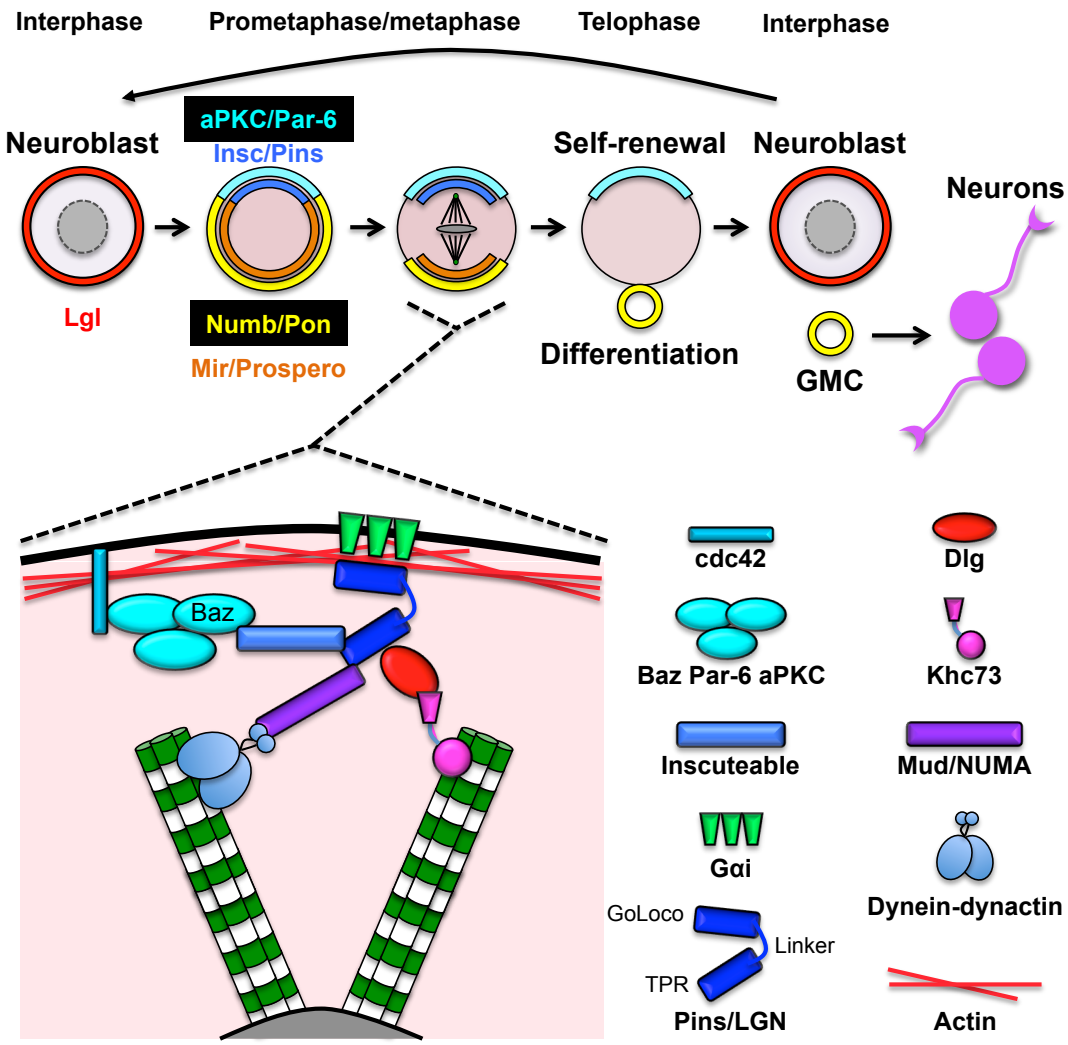

Figure 1.6 Spindle orientation in asymmetric division

(Top) Asymmetric cell division in *Drosophila* neuroblasts results in daughter cells with different cell fates, due to the unequal inheritance of cell fate determinants like Numb. One cell undergoes self-renewal, and the other further differentiates to generate neurons of the nervous system.
(Bottom) Mechanisms of spindle orientation. Two main pathways orient the mitotic spindle: Gαi-Pins-Mud (left microtubule), and Pins-Dlg-Khc73 (right microtubule). For simplicity, the competitive binding of Inscuteable and Mud to Pins is ignored, but note that Inscuteable recruits Pins and then hands off to Mud for spindle anchoring to the cortex. Similar pathways are proposed to exist in symmetric division, but in the absence of Inscuteable, which is neuroblast-specific. See text for full details. Adapted from (Siller and Doe, 2009).

1.4.1.2 Spindle orientation

To ensure the segregation of fate determinants into only one daughter cell, division must occur in the axis of apico-basal polarity. Proteins responsible for this orientation tend to be localised apically, whilst basally localised proteins are involved in cell fate determination: mutation of apical determinants such as *baz* or *insc* result in randomised orientation of the spindle, but loss of basal proteins has no effect (Kraut et al., 1996; Shen et al., 1997; Siegrist and Doe, 2005; Wodarz et al., 1999). However, loss of aPKC or Par-6 also does not affect spindle orientation in relation to cortical cues (Rolls et al., 2003).

Two pathways are known to regulate spindle orientation: Gα-Pins-Mud, and $Pins^{LINKER}$ (Bergstralh et al., 2013a; Johnston et al., 2009). Gαi is a membrane-associated protein; Mud is a microtubule-associated protein that binds to dynein. When in a tripartite complex, Gα, Pins and Mud thus provide a link from the cortex to the microtubules. Initially, Inscuteable binds to Baz at the apical membrane, and recruits Pins by binding the TPR domain (Parmentier et al., 2000; Schaefer et al., 2000; Wodarz et al., 1999; Yu et al., 2000). Pins then binds to Gαi by its (Pins') Go-LOCO motif (Parmentier et al., 2000; Schaefer et al., 2001; Schaefer et al., 2000; Yu et al., 2000), and Mud binds the TPR domain of Pins (Bowman et al., 2006; Izumi et al., 2006; Siller et al., 2006) to replace Inscuteable (Mauser and Prehoda, 2012; Zhu et al., 2011b). Canoe is a PDZ domain containing protein that can also bind to Pins in vivo (Speicher et al., 2008). Reduction in levels of Pins, Gα, Mud or Canoe result in spindles no longer aligned with the apical complex (Bowman et al., 2006; Izumi et al., 2006; Parmentier et al., 2000; Schaefer et al., 2001; Schaefer et al., 2000; Siller et al., 2006; Speicher et al., 2008; Yu et al., 2000). Although *Drosophila* Mud has not been shown to directly associate with dynein-dynactin, the mammalian and *C.elegans* orthologues, NuMA and LIN-5 respectively, associate with the dynein-dynactin complex and anchor the spindle to the cell cortex (Couwenbergs et al., 2007; Merdes et al., 1996; Nguyen-Ngoc et al., 2007). Consistently, the dynein-dynactin complex is required for spindle orientation in neuroblasts (Siller and Doe, 2008). Thus, it is proposed that Gα-Pins-Mud works by linking spindles to the cortex via dynein-dynactin: microtubule pulling then ensures

that one centrosome is localised apically, thus aligning the spindle with the apico-basal polarity axis.

The second pathway is called the Pins^{LINKER} pathway, and involves Pins, the basolateral tumour suppressor Dlg, and kinesin heavy chain 73 (KHC73) (Johnston et al., 2009). Khc73 associates with microtubule plus-ends and binds to Dlg *in vitro* and *in vivo* (Siegrist and Doe, 2005), whilst Dlg also co-immunoprecipitates with Pins (Bellaiche et al., 2001). It is proposed that Khc73 is transported on microtubules to the cortex, localising Dlg and Pins to the apical spindle pole. Pins is activated by phosphorylation on S436 by Aurora A kinase (Johnston et al., 2009), and this phosphorylation is likely to mediate its interaction with the GUK domain of Dlg (Johnston et al., 2009; Zhu et al., 2014; Zhu et al., 2011a). Interestingly, in an inducible-polarity system the Pins^{LINKER} pathway can (partially) orient spindles in the absence of Mud (Johnston et al., 2009). Additionally, in neuroblasts lacking a functional aPKC/Par-6 complex, Numb and Miranda can still segregate asymmetrically at telophase; probably due to this second pathway, which is dependent on microtubule activity (Siegrist and Doe, 2005; Siller et al., 2006; Siller and Doe, 2009). However, disruption of microtubules does not affect asymmetric protein localisation in wild type cells, suggesting that the Dlg-Pins-KHC pathway may be a back-up pathway (Knoblich, 2010).

It has long been known that mutations in *lgl, dlg* or *brat* can result in brain tumour formation in *Drosophila* (Gateff, 1978; Gateff, 1994). More recent work identifying roles for these genes in asymmetric cell division has hinted at a role in aberrant cell division in tumourigensis (Albertson and Doe, 2003; Knoblich, 2010). Mutations of fate determinants *numb* and *prospero*, cell cycle kinases *aurora* and *polo*, spindle orientation genes, or over-activation of aPKC or Notch, can also lead to tumour formation (Knoblich, 2010). The simplest explanation is a failure to segregate cell fate determinants correctly, and the generation of two neuroblasts as daughter cells. An exponential increase in cell number can then occur. Indeed, the presence of excessive neuroblast numbers in clones of mutant cells, or whole mutants, is a marker for defective asymmetric division.

1.4.2 Symmetric Cell Division and Spindle orientation

Most divisions are symmetric, resulting in two equally sized and polarised daughter cells. The spindle is generally oriented in the plane of the epithelium, perpendicular to the axis of apico-basal polarity. Because of this, it is not so intuitively clear that cell division requires alterations or tight regulation of apico-basal polarity itself, unlike asymmetric division: although the orientation of the spindle is still important.

Spindle orientation in symmetric division employs many of the same mechanisms as in asymmetric division (Morin and Bellaiche, 2011). Both Pins and Mud are required for correct orientation, and a recent study has identified Dlg as also being important – probably through its binding to Pins, as in neuroblasts (Bergstralh et al., 2013b). Scribble, another member of the basolateral polarity protein complex, has also been implicated in spindle orientation, though the mechanism is unclear (Nakajima et al., 2013). A recent study has also suggested that aPKC is important for spindle orientation, possibly by removing Pins from the apical membrane in mitosis (Guilgur et al., 2012). aPKC is reported as being able to phosphorylate LGN, the mammalian Pins homologue, which removes LGN from the apical surface of mitotic cells (Hao et al., 2010). Once removed from the apical membrane, Pins presumably then is able to promote spindle orientation in conjunction with Dlg. However, in other systems, aPKC is not required for spindle orientation (Bergstralh et al., 2013b; Peyre et al., 2011); and neither affecting aPKC nor mutating the putative aPKC phosphorylation site in LGN affect LGN's localisation (Peyre et al., 2011). Additionally, in follicle cells, aPKC is absent from the apical membrane during mitosis and may not be present to phosphorylate Pins (Bergstralh et al., 2013b). Thus whilst good evidence exists for Dlg's involvement in spindle orientation, data supporting or explaining roles for aPKC or Scribble remains limited. Nonetheless, recent work has suggested novel roles in spindle orientation for a number of polarity proteins.

In asymmetric cell division, misoriented mitotic spindles can result in division not being aligned with the polarity axis, and therefore cell fate determinants not being correctly segregated into daughter cells (Siller and Doe, 2009). In symmetric division, the orientation of the mitotic spindle has been proposed to be important for

the maintenance of the epithelial layer, and – albeit controversially – that misorientation could lead to cells escaping the epithelium and aiding in tumour formation/progression (Nakajima et al., 2013). Symmetrically dividing cells divide in the plane of the epithelium: misoriented spindles might therefore lead to daughter cells establishing either above or below the epithelial layer. Disruption of the planar spindle alignment is proposed to cause epithelial to mesenchymal transition and cancer, by escaping the epithelial layer. Recent work has suggested that misalignment of the spindle is correlated with cell delamination and cell death in the wing disc epithelium: and preventing cell death can lead to the formation of tumour-like structures (Guilgur et al., 2012; Nakajima et al., 2013). In another tissue, the follicle cell epithelium, misoriented spindles can result in cells transiently leaving the epithelial layer, but they can reinsert themselves without dying (D.Bergstralh and D. St Johnston, unpublished). The contribution of spindle misorientation to tumour formation, whilst attractive, may thus require further investigation.

Finally, consistent with the evolving views of the roles of polarity determinants, LLGL2, the human homologue of Lgl, was reported as being involved in spindle assembly in MDCK cells (Yasumi et al., 2005). Lgl was reported as directly binding to both LGN (Pins) and NuMA (Mud), and functions by strengthening the interaction between these proteins. RNAi of LLGL2 or LGN resulted in spindle disorganisation, multipolar spindles, and multi-nucleated cells (Yasumi et al., 2005). However, roles for LGN/Pins or NuMA/Mud in spindle assembly, as opposed to orientation, have not been well studied.

1.5 Polarity and cancer

Some 90% of human cancers are derived from epithelial cells (Macara and McCaffrey, 2013), which exhibit organised cell and tissue structures, regulated spindle orientation, and the presence of adherens junctions between cells. Loss of polarity results in perturbation of these features, which is also seen in the tissue disorganisation associated with cancer. An important hallmark of more advanced and invasive tumours is the loss of epithelial character and appearance of mesenchymal-like cells: epithelial-mesenchymal transition (EMT). Loss of cell-cell adhesion and apico-basal polarity are both hallmarks of EMT, and whilst EMT is accepted as an important step in (late) tumour progression (Wodarz and Nathke, 2007), the involvement of polarity proteins in tumourigensis is less developed. Each of the polarity complexes has been suggested as contributing to many of the steps in tumourigensis, and these are summarised below.

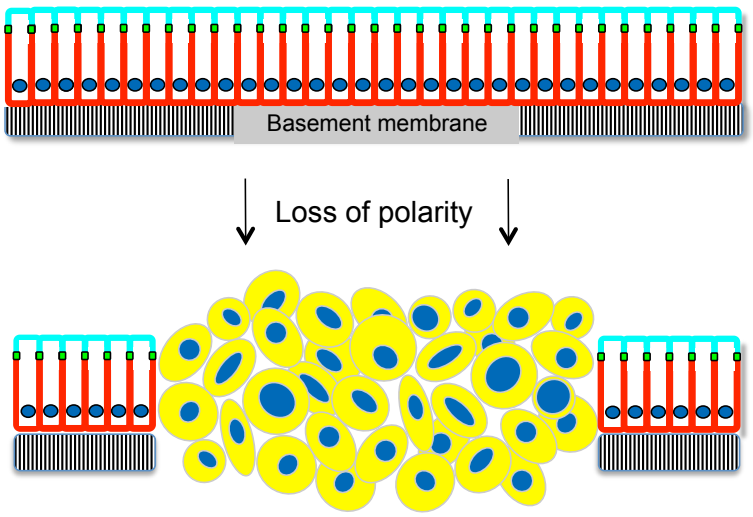

Figure 1.7 Polarity and Cancer

Loss of polarity results in a disorganised, amorphous mass of cells rather than a functional epithelial layer, and is proposed to contribute to tumour formation and progression. Many polarity determinants are implicated in tumourigensis and are affected in human cancers, though the mechanistic details and direct causes are still relatively unknown. Adapted from (Lee and Vasioukhin, 2008).

1.5.1 Polarity complexes

The basolateral polarity proteins Dlg and Lgl were identified in screens for mutations causing overgrowth in the imaginal discs of *Drosophila* larvae over 30 years ago (Gateff, 1978). Scribble, which along with Dlg and Lgl comprises the Scribble module, was similarly identified more recently (Bilder et al., 2000). Mutation in any of these genes leads to loss of polarity and hyperproliferation of imaginal discs in homozygous mutant larvae. Cells in these larvae actually proliferate slower than wild-type compatriots – such that mutant discs at early stages are smaller than homozygous discs – but larvae fail to pupate, and the discs continue to grow for a number of additional days (Froldi et al., 2008; Menendez et al., 2010; Woods and Bryant, 1989). The best-studied functions of the Scribble module members are in apico-basal polarity, but more roles have also been suggested in asymmetric cell division (Dlg and Lgl (Albertson and Doe, 2003)), spindle orientation (Dlg (Bergstralh et al., 2013b), Scribble (Nakajima et al., 2013), and in this work, Lgl), the cell cycle (Scrib, Dlg, Lgl) (Brumby and Richardson, 2003), planar cell polarity and migration (Scribble (Elsum et al., 2012; Ezan and Montcouquiol, 2013; Montcouquiol et al., 2003).

Scribble has been observed to be down regulated or mislocalised in tumours in mice and humans: in cervical cancer, adenocarcinoma in the colon and prostate cancer (Ellenbroek et al., 2012). Scribble and Dlg1 are down regulated in cervical cancer: with stronger effects at later, more invasive stages. Viral oncoproteins can also interact with Dlg1 and Scribble. In mammalian cells, HPV E6 oncoproteins promote ubiquitin-mediated degradation of Scribble (Thomas et al., 2005), and Scribble protein levels are reduced in HPV-positive cervical tumours (Ellenbroek et al., 2012). Dlg expression levels and localisation are also altered in colon cancers, cervical cancers, and invasive squamous carcinomas (Huang and Muthuswamy, 2010). Lgl1 transcripts are absent in tumours from breast, prostate, lung, ovarian and colon carcinomas (Grifoni et al., 2004); and Lgl2 localisation is affected in gastric carcinomas (Lisovsky et al., 2009).

In *Drosophila*, a single mutation is able to cause loss of polarity and neoplasia in discs, but only in whole mutant animals (Bilder, 2004; Bilder et al., 2000).

Homozygous mutant clones of *dlg*, *lgl* or *scribble*, generated in a heterozygous background, are eliminated by apoptosis (Froldi et al., 2010; Menendez et al., 2010). The expression of oncogenes like Ras can lead to tumour formation, but rarely spreading into other tissues (Bilder et al., 2000; Brumby and Richardson, 2003; Humbert et al., 2003). However, the expression of Ras in conjunction with loss of polarity proteins leads to large tumours and extensive spreading (Brumby and Richardson, 2003; Pagliarini and Xu, 2003; Wu et al., 2010). Similarly, prevention of cell death combined with loss of polarity leads to large tumours.

In humans, tumour progression is a multistep process, and individual mutations hardly ever lead to tumour formation: even though Ras is expressed in ~30% of human cancers, co-operation with other oncogenes or loss of tumour suppressors – like Scribble - is required for tumour progression and invasive potential (Ellenbroek et al., 2012; Huang and Muthuswamy, 2010).

The apical polarity proteins have also been linked to cancer progression (Macara and McCaffrey, 2013). Dlg, Lgl and Scribble were identified as co-operating with Ras in a screen to look for mutations in genes that could promote tumour invasion and growth in a Ras-expression background (Pagliarini and Xu, 2003). Apical polarity proteins Baz, Stardust and Cdc42 were also identified (Pagliarini and Xu, 2003). Elevated expression of PKCι, a human aPKC homologue, was found in lung, colon and ovarian cancer, and was associated with Ras activity (Eder et al., 2005; Murray et al., 2004; Regala et al., 2005). PTEN is a tumour suppressor frequently mutated in human cancers: it has also been found to associate with the aPKC/Par-6 complex in mammals and *Drosophila* (Wodarz and Nathke, 2007). Furthermore, LKB1, the mammalian homologe of *C.elegans* Par-4, which associates with aPKC and Par-6, is implicated in the development of and predisposition to some cancers (Wodarz and Nathke, 2007). Par-3 has recently been suggested as having tumour suppressive properties, and loss of Par-3 results in increased incidence of some cancers (McCaffrey et al., 2012). Par-6 is over-expressed in human breast cancer (Nolan et al., 2008), and might aid in EMT through interaction with TGF-beta, which is involved in the dissolution of tight junctions and epithelial integrity (Ellenbroek et al., 2012).

The contribution of the Crumbs complex is less well studied. Loss of Crb3 is observed in IBML (Immortalised Baby Mouse Kidney) and BRK (Baby Rat Kidney) cells that exhibit tumour potential, and recent work has identified factors involved in EMT, Snail and ZEB1, as being able to repress Crb levels (Ellenbroek et al., 2012; Huang and Muthuswamy, 2010). PATJ, a member of the Crumbs complex, can be targeted for degradation by the HPV E6 oncoproteins in cancer-derived cell lines, similar to Scribble and Dlg (Storrs and Silverstein, 2007; Thomas et al., 2005). PATJ is required for tight junction formation, and its degradation might also lead to cancer progression (Ellenbroek et al., 2012).

1.5.2 Polarity and The Hippo Pathway

Recently, polarity proteins have been linked to the Hippo signalling pathway (Genevet and Tapon, 2011; Grzeschik et al., 2010b) The Hippo pathway is a conserved pathway that controls cell growth, proliferation and cell survival, and was originally identified in *Drosophila* (Halder and Johnson, 2011; Pan, 2010). The key kinases in the pathway, Hippo and Warts, are both tumour suppressors, and mutation in either leads to dramatic over-proliferation. Hippo and Warts are involved in a kinase cascade to regulate the phosphorylation and localisation of the transcriptional co-activator Yorkie. When Hippo signalling is active, Yorkie is phosphorylated and sequestered into the cytoplasm by 14-3-3 proteins. When Hippo signalling is inactive, Yorkie is able to enter the nucleus where it acts with its binding partner Scalloped to activate target genes involved in cell proliferation and survival, such as *cyclin E* and *diap1* (Huang et al., 2005). Mutations in *yki* lead to tissue undergrowth, and over-expression of *yki* results in massive overproliferation similar to *hippo* or *warts* mutants (Halder and Johnson, 2011; Huang et al., 2005). Recently, Lgl, aPKC and Crumbs have been linked to Hippo signalling. aPKC acts as inhibitor of Hippo signalling by reducing the apical localisation of Hippo, and mediating interaction with the Hippo negative regulator RASSF (Grzeschik et al., 2010b; Parsons et al., 2010; Polesello and Tapon, 2007). Overexpression of aPKC is reported as leading to upregulation of diap1 or expanded, although no effect on tissue overgrowth was seen (Grzeschik et al., 2010a). Consistent with its behaviour in antagonising aPKC activity, Lgl promotes Hippo signalling, and loss of *lgl* is associated with increased proliferation and cell survival via Hippo signalling and the

upregulation of Yki target genes (Grzeschik et al., 2010a; Grzeschik et al., 2010b; Parsons et al., 2010). Hippo pathway target genes are also reportedly upregulated in *dlg* or *scrib* mutant clones (Doggett et al., 2011; Verghese et al., 2012; Zhao et al., 2008). In zebrafish, Scribble interacts with YAP (vertebrate Yki) and suppresses YAP activity (Skouloudaki et al., 2009); knockdown of Scribble also results in reduced YAP phosphorylation in mammalian cells (Mohseni et al., 2014). Loss of Crumbs results in Yki-dependent Hippo pathway target gene upregulation (Chen et al., 2010; Ling et al., 2010; Richardson and Pichaud, 2010; Robinson et al., 2010). Crumbs binds to Expanded, a FERM protein and upstream Hippo pathway regulator, and promotes Expanded apical localisation (Chen et al., 2010; Ling et al., 2010; Robinson et al., 2010). Additionally, members of the Crumbs complex are able to bind to YAP/TAZ in cell culture, and knockdown of PALS1 or CRB3 (mammalian homologues of Stardust and Crumbs) leads to a decrease in phospho-YAP levels (Varelas et al., 2010).

1.5.3 Asymmetric cell division and cancer

As highlighted above, many of the proteins involved in polarity regulation are also involved in the orientation of the mitotic spindle and asymmetric cell division. Brain tumours can be induced by perturbing the regulation of spindle orientation, or by mutations in the polarity determinants themselves. Lgl, Dlg and aPKC (Albertson and Doe, 2003; Peng et al., 2000); Numb, Prospero and Brat (Bello et al., 2006; Betschinger et al., 2006; Bowman et al., 2008; Lee et al., 2006c); Aurora A (Lee et al., 2006a; Wang et al., 2006); Polo (Wang et al., 2007); Notch (Wang et al., 2006); and Pins and Mud (Bowman et al., 2006; Izumi et al., 2006; Siller et al., 2006) have all been implicated in tumour formation and/or altered neuroblast numbers, indicative of a role for asymmetric cell division. Asymmetric cell division is also present in a number of human tissues, such as the brain, skin, gut, mammary glands and haematopoietic system (Knoblich, 2010). Although many of the players in *Drosophila* and *C.elegans* are present in humans, their functions are not always the same. However, asymmetric division in stem cells from mouse mammary glands is perturbed in tumours, and Numb is no longer localised into just one of the daughter cells upon division (Knoblich, 2010). An inhibitor of Numb also results in the initial divisions in the development of the haematopoietic system occurring as

symmetric rather than asymmetric, which might contribute to the progression of chromic myeloid leukaemia (Knoblich, 2010; Wu et al., 2007).

1.5.4 E-Cadherin and EMT

Polarity proteins help to position the adherens junctions, and cell polarity is reciprocally dependent on the establishment of the junctions (Knust and Bossinger, 2002; St Johnston and Ahringer, 2010). E-Cadherin is a key component of adherens junctions, and loss of E-Cadherin is a hallmark of cancer progression and more invasive tumours. Particularly, the loss of E-Cadherin is a hallmark of epithelial-mesenchymal transition (EMT), and is associated with the loss of intercellular junctions and cells detaching and escaping from the epithelial layer (Cavallaro and Christofori, 2004; Lamouille et al., 2014; Wodarz and Nathke, 2007). E-Cadherin has a strong reputation as a tumour suppressor gene, and is particularly associated with the progression from adenoma to carcinoma, and metastatic potential. Two transcription factors, Twist and Snail, were identified as being able to induce EMT in *Drosophila*: causing epithelial cells from the embryonic ectoderm to form mesoderm (Leptin, 1991). A key effect of Twist and Snail is to repress E-Cadherin expression, and their levels are upregulated in many cancers (Leptin, 1991; Micalizzi et al., 2010). In colorectal cancers, only late stage tumours display this EMT and E-Cadherin repression, suggesting these might be some of the later steps, rather than important for initial tumour formation (Bell and Thompson, 2014; Chaffer and Weinberg, 2011). Adenocarcinomas – non-invasive tumours – maintain E-Cadherin expression and levels even as the epithelium becomes disorganised (Bell and Thompson, 2014). The polarity determinants in *Drosophila* are required for localising E-Cadherin, and loss of polarity may therefore be a more basic step in tumour formation than EMT and loss of cell-cell adhesion.

1.6 Aims of the PhD project

Apico-basal polarity is a key feature of epithelial cells, and is important for cell function and tissue integrity, and misregulation is thought to be important in tumour formation and progression. As cells enter mitosis, they undergo cell rounding and interkinetic nuclear migration, important events that involve dramatic morphological changes. Polarity proteins have been studied in asymmetric cell division fairly intensively, focussing on spindle orientation and cell fate establishment. Recent work has also indicated functions for Scrib, Dlg and aPKC in symmetric division. We were interested to look carefully at cell polarity through mitosis, initially using the wing disc epithelium as a model, since it exhibits some of the more dramatic cell shape and IKNM changes. Work from mouse models has shown that planar polarity determinants can be internalised in mitosis, and relocalise to their interphase positions after mitosis with the aid of their neighbouring cells (Devenport et al., 2011): the net effect being to preserve polarity for the cell(s). We wondered whether any similar effects might occur in *Drosophila*. Additionally, recent studies highlighted the need to look carefully, because polarity mutants have very dramatic effects that could over-shadow any other, subtler, roles they might play. We would then see whether changes we had observed would have roles in asymmetric division as well, and look at the conservation of regulation between these systems. Finally, we would consider any link between mitosis, polarity and cancer.

Chapter 2. Materials and Methods

2.1 *Drosophila* genetics

2.1.1 Target gene expression: the GAL4/UAS system

The GAL4/UAS system is a powerful tool that is used to express gene products under the control of a specific promoter: it can be used to overexpress genes or RNAi, or to label particular cells or tissues (Brand and Perrimon, 1993). GAL4 is a transcription factor from yeast that binds to an **U**pstream **A**ctivation **S**equence (UAS) and activates genes located subsequent to that UAS. Any particular gene can be cloned under the control of a UAS, and transgenic flies containing the UAS.X sequence (for gene *X*) can be generated. Transgenic flies can also be created to express the GAL4 construct under the control of a specific tissue promoter. Crossing these GAL4 flies to UAS.X flies results in the expression of X in a specific region or tissue (Figure 2.1). For example, some GAL4 drivers used in this study were: hedgehog.GAL4, expressed only in the posterior compartment of the wing (Figure 2.1); MS1096.GAL4, expressed in the entire wing compartment; and patched.GAL4, expressed in a stripe down the centre of the wing disc.

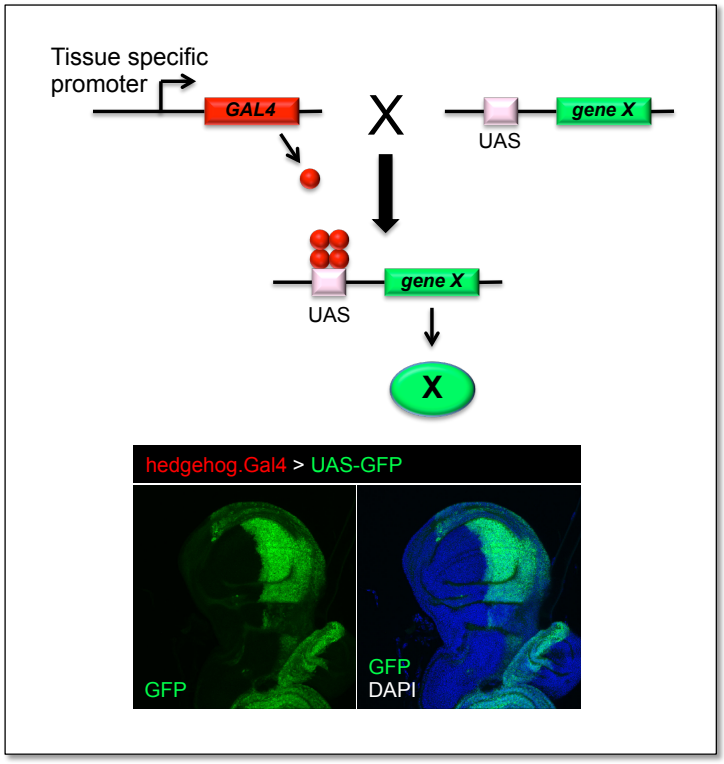

Figure 2.1 The GAL4/UAS system in *Drosophila*

2.1.2 Generation of mitotic clones: the FLP/FRT and MARCM systems

Animals that are homozygous mutants for many genes fail to develop beyond the early stages of the *Drosophila* life cycle. The FLP/FRT system is an elegant method to overcome this, by generating mitotic clones of homozygous mutant cells within heterozygous mutant tissue (Perrimon, 1998). The system, illustrated in Figure 2.2, uses the (yeast) site-specific FLP recombinase, which catalyses recombination events between FRT (**F**LP **R**ecombination **t**arget) sites. The system is often used with the FLP recombinase under the control of a heat-shock inducible promoter, where the recombinase is expressed on subjecting transgenic flies to a heat shock. The timing, length and temperature of the heat shock can thus provide a reliably consistent method of generating clones of mutant cells.

In the FLP/FRT system, the mutation one wishes to study must be recombined with an FRT site in the same chromosome arm. A common bank of widely used FRT sites is available. The mutant stock is crossed to a line containing the same FRT site, in addition to a ubiquitously expressed GFP promoter and the wild type allele of the mutant gene. The presence of hsFLP is also required in the progeny. Upon heat-shock, FLP is expressed and catalyses recombination between the FRT sites of homologous chromosomes: resulting in chimeric chromosomes consisting of two sister chromatids, one carrying the mutant allele and one the wild type allele.

In the next mitosis, the sister chromatids segregate into the daughter cells producing two possible outcomes. In the first, daughter cells inherit one chromatid with the wild type allele (and GFP), and one chromatid with the mutant allele: both daughters are like other cells in the tissue, being heterozygous for the mutation and expressing one copy of GFP. In the second outcome, one daughter cell inherits both of the chromatids carrying the wild type allele and GFP, and the other daughter cell inherits both chromatids containing the mutant allele and no GFP. The clone therefore manifests as GFP-negative cells, which are homozygous for the mutant allele; and cells homozygous for the wild type allele, which are doubly GFP-positive (“twin-spot”). The remaining tissue is heterozygous for the mutation and expresses one copy of GFP.

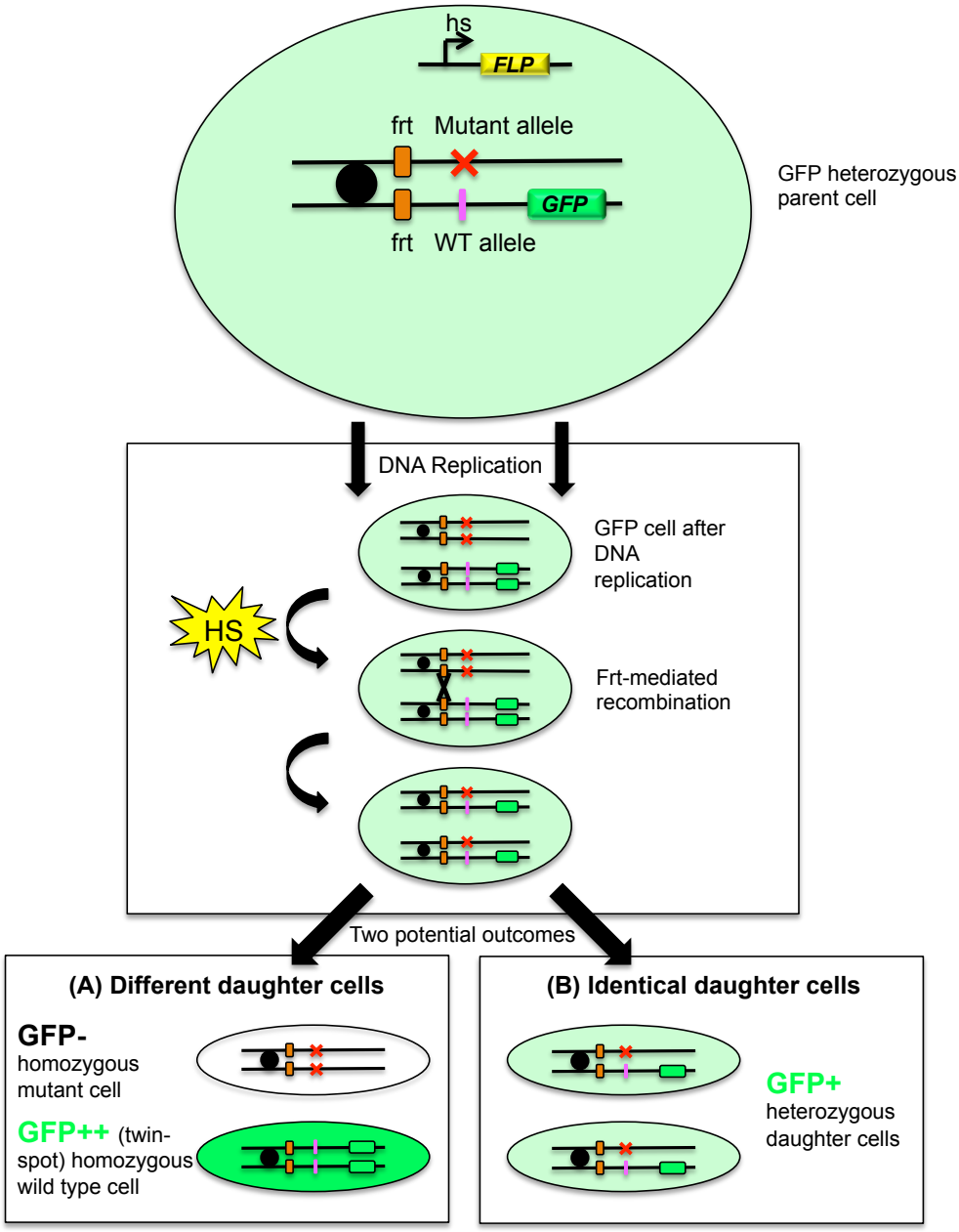

Figure 2.2 The FLP/FRT system

Upon heat-shock, FLP recombinase is expressed and catalyses recombination between FRT sites. In the next mitosis, sister chromatids segregate producing 2 possible outcomes: (A) two different daughter cells, one homozygous for the wild type allele and expressing two copies of GFP ("twin-spot"), and one homozygous for the mutant allele with no GFP expression; (B) Two identical daughter cells, heterozygous for the mutant allele and each expressing one copy of GFP. Homozygous mutant cell clones are therefore marked by the absence of GFP. See text for further details.

The MARCM system (**M**osaic **A**nalysis with a **R**epressible **C**ell **M**arker) is a variant of the standard FLP/FRT technique, and results in mutant clones marked positively with GFP expression, in contrast to the negative marking of such clones using FLP/FRT (Lee and Luo, 1999). In this system, all cells ubiquitously express GAL80, which is a repressor of GAL4. Before recombination events, all cells express both GAL4 and GAL80 and the UAS.GFP transgene is not expressed. Upon heatshock-induction of FLP expression and FRT recombination, the GAL80 transgene is not present in homozygous mutant cells. Thus, GAL4 is active in these cells, which then express the UAS.GFP transgene: mutant cells are marked by the presence of GFP.

Clones in wing disc cells were typically generated with a 1-hour heat shock of 37C 60 hr (+/-12 hours) after egg laying, and larvae were dissected at the third instar stage. Clones in ovarian follicle cells were generated by 2 x 1-hour heat shocks of adult females, which were dissected ~5 days after heat shock. To generate larger clones, third instar larvae were subject to heat shock and the resulting adult females dissected. Expression of UAS.Lgl-GFP, UAS.myrLgl-GFP or UAS.LglASA-GFP in FRT wild type or FRT mutant backgrounds was achieved using the MARCM system (replacing the usual UAS.GFP with an Lgl construct).

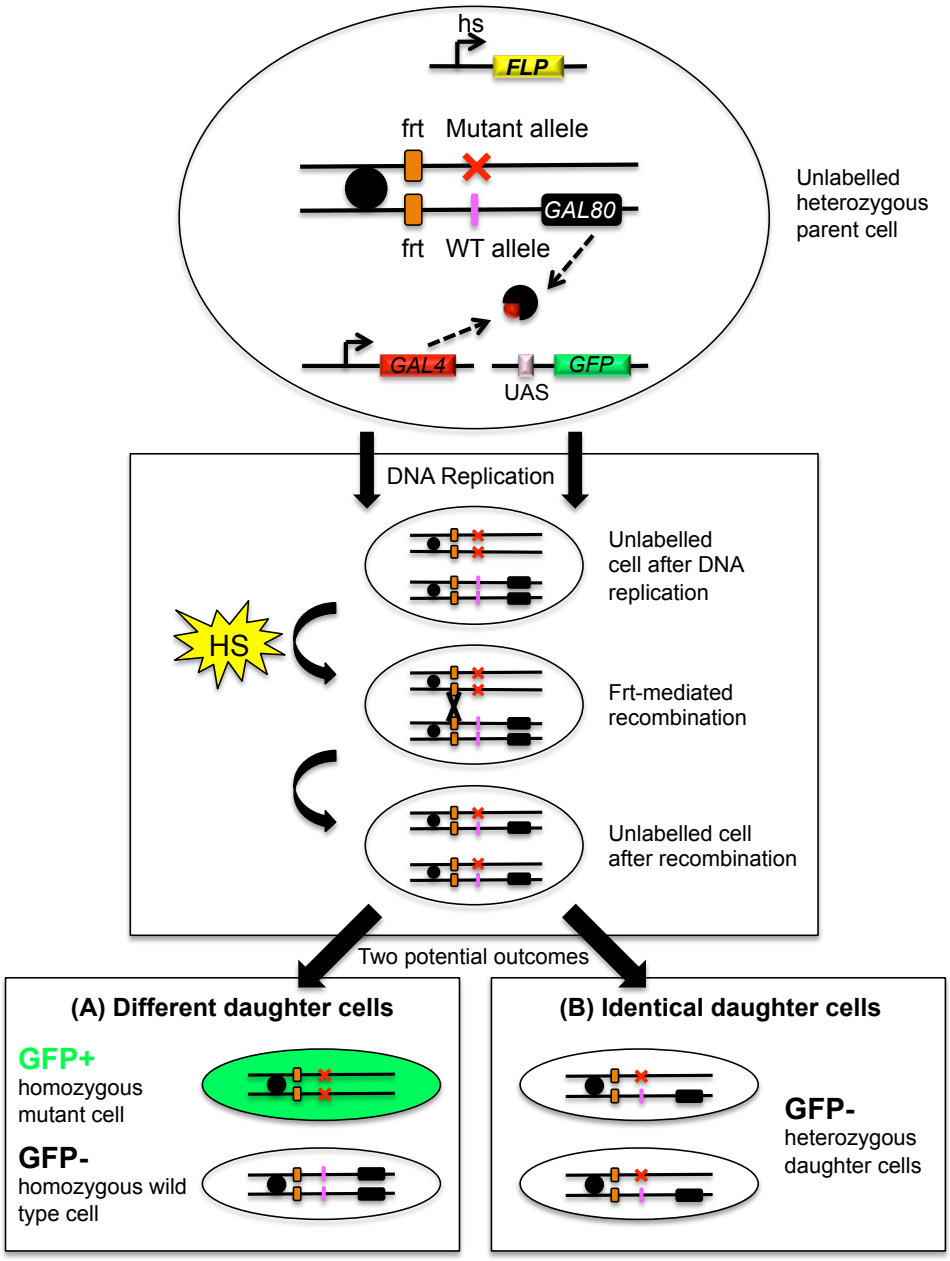

Figure 2.3 The MARCM system

The MARCM system is similar to FLP/FRT (Figure 1.2), but results in homozygous mutant cells being marked positively with GFP. Every cell expresses GAL80, a repressor of GAL4, on the chromosome arm containing the FRT site: GFP expression is normally inhibited. On heat-shock induced recombination and chromatid segregation, homozygous mutant cells formed do not carry the GAL80 transgene. GAL4 expression is therefore not repressed, and these mutant cells express GFP.

2.1.3 *Drosophila* strains and genotypes

The *aPKC kinase-dead* alleles *FRT42B aPKC^{psu265}*, *FRT42B aPKC^{psu141}* and *FRT42B aPKC^{psu417}* were gifts from A. Wodarz (Kim et al., 2009). *FRT82B aurora A^{14641}* and *aurora A^{8839}* were gifts from C. Doe (Lee et al., 2006a). E-Cad-GFP and Crb-GFP were gifts from Y. Hong (Huang et al., 2009). UAS.Baz-mCherry was a gift from T. Harris (McGill et al., 2009). UAS.Lgl-GFP and UAS.Lgl3A-GFP were gifts from W. Deng (Tian and Deng, 2008). UAS.AuroraA-GFP was a gift from J. Knoblich (Berdnik and Knoblich, 2002). UAS.E-Cad7 was a gift from J-P. Vincent. ubi.His2B-RFP; ubi.Jup-GFP was a gift from P. Gaspar, originating from ubi.His2B-RFP (gift from Y. Bellaiche) and ubi.Jup-GFP (Karpova et al., 2006). UAS.AuroraB-RNAi was from the KK library.

Other fly strains are available from Bloomington Drosophila Stock Centre and described in FlyBase (Ashburner and Drysdale, 1994; Tweedie et al., 2009). Strains we used from previous studies were: *aurora A^{87Ac-3}* (Glover et al., 1995), Arm-GFP (McCartney et al., 2001), UAS.Par-6-GFP (David et al., 2010), UAS.Dlg-GFP (Koh et al., 1999), *aPKC^{K06403}* (Wodarz et al., 2000), *lgl^{334}* described in (Mechler et al., 1985), *dlg^{M52}* (Woods and Bryant, 1991), UAS.E2F and UAS.Dp (Neufeld et al., 1998), *baz^{4}* (Muller and Wieschaus, 1996), *shg^{R69}* (Godt and Tepass, 1998), *scrib^{2}* (Bilder and Perrimon, 2000), *crb$^{82-04}$* (Ling et al., 2010), *lgl^{4}* (Gateff, 1978), UAS.Ras^{V12} (Karim and Rubin, 1998), UAS.yki-nls (Sidor et al., 2013), UAS.Scrib-GFP (Zeitler et al., 2004) and sqh.sqh-GFP (Royou et al., 2002). UAS.E-Cad7, UAS.E-Cad5 and UAS.E-Cad9 were gifts from J-P.Vincent.

Of the key strains used, *aurora A^{87Ac-3}*, *aPKC^{K06403}* and *lgl^{4}* are null alleles; *Df(3r)Exel6163* has a deficiency covering the *aurora A* gene; *aurora A^{14641}* has a point mutation in the activation loop and shows comparable phenotypes to *aurora A null* mutants; *aPKC^{psu265}*, *aPKC^{psu141}* and *aPKC^{psu417}* are kinase-dead alleles; and *lgl^{334}* is hypomorphic.

UAS.myrLgl-GFP was generated in our lab by R.Brain. UAS.LglASA, UAS.LglASA-GFP, UAS.AurA^{T295E} and UAS.AurA^{T295D} were generated for this study.

Genotypes in figures are as follows. Wild type samples were yw and not listed below:

Figure 2.1	*w;; hh.GAL4/UAS-GFP*
Figure 3.1 A, E, G	*w;; Crb-GFP (knock-in line)*
Figure 3.1 B, D	*MS1096.GAL4/+ ;; UAS.Par-6-GFP/+*
Figure 3.1 C	*MS1096.GAL4/+ ; UAS.Baz-mCherry/+*
Figure 3.1 H	*E-Cad-GFP*
Figure 3.2 A	*MS1096.GAL4/+ ;; UAS.Dlg-GFP/+*
Figure 3.2 B	w;; *Scrib-GFP (knock-in line)*
Figure 3.2 C-E	*MS1096.GAL4/+ ;; UAS.Lgl-GFP/+*
Figure 3.2 D-E	*yw, Actin>CD2>GAL4, hs.FLP/+;; UAS.Lgl-GFP*
Figure 3.2 F	*tub.GAL4/+ ;; UAS.Lgl-GFP/+*
Figure 3.3 C	*yw, hs.FLP/+ ; FRT40A lgl^{4}/FRT40A GFP*
Figure 3.4 A, G	*tub.GAL4/+ ;; UAS.Lgl-GFP/+*
Figure 3.4 B, J	*tub.GAL4/+ ;; UAS.Lgl3A-GFP/+*
Figure 3.4 C, F, H	*yw, tub.GAL4, hs.FLP/+ ; FRT42B $aPKC^{417}$/FRT42B CD21 y+ GAL80; UAS.Lgl-GFP/+*
Figure 3.4 D, I	*yw, tub.GAL4, hs.FLP/+ ; FRT42D $aPKC^{K06403}$/FRT42D CD21 y+ GAL80; UAS.Lgl-GFP/+*
Figure 3.4 E	*MS1096.GAL4/+ ;; UAS.Lgl3A-GFP/+*
Figure 3.5 A	*yw, hs.FLP/+ ; FRT40A/FRT40A GFP*
Figure 3.5 B	*yw, hs.FLP/+ ; FRT42D $aPKC^{K06403}$/FRT42D*
Figure 3.5 C	*yw, tub.GAL4, hs.FLP/+ ; FRT42B $aPKC^{417}$/FRT42B CD21 y+ GAL80; UAS.Lgl-GFP/+*
Figure 3.5 D	*yw, tub.GAL4, hs.FLP/+ ; FRT42B $aPKC^{265}$/FRT42B CD21 y+ GAL80; UAS.Lgl-GFP/+*
Figure 3.6 A	*MS1096.GAL4/+ ;; UAS.Lgl-GFP/+*
Figure 3.6 B	*MS1096.GAL4/+ ; UAS.$aPKC^{CAAX}$/+; UAS.Lgl-GFP/+*
Figure 3.6 C	*ptc.GAL4, UAS.GFP/UAS.$aPKC^{CAAX}$*
Figure 3.7 A, C-E	*MS1096.GAL4/+ ;; UAS.Lgl-GFP/+*
Figure 3.7 B	*en.GAL4/+; UAS.Lgl-GFP/+;aurora A^{87Ac-3}/Df(3R)Exel6161*
Figure 3.9 A	*ptc.GAL4, UAS.GFP/UAS.Aurora A^{T295D6}*
Figure 3.9 B-C	*ptc.GAL4, UAS.GFP, UAS.Bora/UAS.Aurora A^{T295D6}*

Figure 3.10 A	*MS1096.GAL4/+ ;; UAS.Lgl-GFP/+*
Figure 3.10 B	*en.GAL4/+ ; UAS.Lgl-GFP/+;aurora A^{87Ac-3}/Df(3R)Exel6161*
Figure 3.10 C	*MS1096.GAL4/+ ; UAS.Aurora B RNAi*
Figure 3.10 D	*MS1096.GAL4/+ ;; UAS.Lgl-GFP/+*
Figure 3.10 E,F	*ptc.GAL4, UAS.GFP/UAS.Aurora B RNAi*
Figure 3.11 A,F	*yw, tub.GAL4, hs.FLP, UAS.nucGFP-myc/+ ;; FRT82B/FRT82B CD21 y+ GAL80*
Figure 3.11 B,G	*yw, tub.GAL4, hs.FLP, UAS.nucGFP-myc/+ ;; FRT82B aurora A^{14641}/FRT82B CD21 y+ GAL80*
Figure 3.11 C,H	*yw, tub.GAL4, hs.FLP, UAS.nucGFP-myc/+ ; UAS.Aurora B RNAi/+; FRT82B/FRT82B CD21 y+ GAL80*
Figure 3.11 D-E,I	*yw, tub.GAL4, hs.FLP, UAS.nucGFP-myc/+ ; UAS.Aurora B RNAi/+ ; FRT82B aurora A^{14641}/FRT82B CD21 y+ GAL80*
Figure 3.11 J	*ptc.GAL4, UAS.GFP/+*
Figure 3.11 K	*ptc.GAL4, UAS.GFP/+ ; aurora A^{14641}/aurora A^{87Ac-3}*
Figure 3.11 L	*ptc.GAL4, UAS.GFP/UAS.Aurora B RNAi*
Figure 3.11 M-N	*ptc.GAL4, UAS.GFP/UAS.Aurora B RNAi; aurora A^{14641}/aurora A^{87Ac-3}*
Figure 3.12 A, E	*MS1096.GAL4/+ ;; UAS.LglASA-GFP/+*
Figure 3.12 C	*MS1096.GAL4/+ ;; UAS.Lgl-GFP/+*
Figure 3.12 D	*MS1096.GAL4/+ ;; UAS.Lgl3A-GFP/+*
Figure 3.12 F	*TJ.GAL4/UAS.Lgl-GFP*
Figure 3.12 G	*TJ.GAL4/UAS.Lgl3A-GFP*
Figure 3.12 H, I	*TJ.GAL4/UAS.LglASA-GFP*
Figure 4.1 A-B	*MS1096.GAL4/+ ;; UAS.myrLgl-GFP/+*
Figure 4.1 C	*tub.GAL4/UAS.myrLgl-GFP*
Figure 4.1 D	*TJ.GAL4/UAS.myrLgl-GFP*
Figure 4.2 B, F-G	*lgl^{4}/lgl^{334}*
Figure 4.2 E	*dlg^{M52}/dlg^{1}*
Figure 4.3 B	*dlg^{M52}/dlg^{1}*
Figure 4.3 C	*lgl^{4}/lgl^{334}*
Figure 4.4 A	*ubi.His2B-RFP, ubi.Jup-GFP/Tm6b*
Figure 4.4 B-C	*lgl^{4}/lgl^{334} ; ubi.His2B-RFP, ubi.Jup-GFP/+*

Figure 4.5 B, F-G	*lgl^{4}/lgl^{334}*
Figure 4.6 A, D, H	*yw, tub.GAL4, hs.FLP/+ ; FRT40A lgl^{4}/FRT40A CD21 y+ GAL80; UAS.Lgl-GFP/+*
Figure 4.6 B, E, I	*yw, tub.GAL4, hs.FLP/+ ; FRT40A lgl^{4}/FRT40A CD21 y+ GAL80; UAS.myrLgl-GFP/+*
Figure 4.6 C, F, J	*yw, tub.GAL4, hs.FLP/+ ; FRT40A lgl^{4}/FRT40A CD21 y+ GAL80; UAS.LglASA-GFP/+*
Figure 4.7 A, F	*lgl^{4}/lgl^{334} ; hh.GAL4/UAS.Lgl-GFP*
Figure 4.7 B, F	*lgl^{4}/lgl^{334} ; hh.GAL4/UAS.myrLgl-GFP*
Figure 4.7 C-E, F	*lgl^{4}/lgl^{334} ; hh.GAL4/UAS.LglASA-GFP*
Figure 4.7 F	*mud^{1}/mud^{4}*
Figure 4.8 A	*yw, tub.GAL4, hs.FLP, UAS.nucGFP-myc/+ ;; FRT40A lgl^{4}/FRT40A CD21 y+ GAL80*
Figure 4.8 B, F-G	*yw, tub.GAL4, hs.FLP/+ ; FRT40A lgl^{4}/FRT40A CD21 y+ GAL80; UAS.Lgl-GFP/+*
Figure 4.8 C, I	*yw, tub.GAL4, hs.FLP/+ ; FRT40A lgl^{4}/FRT40A CD21 y+ GAL80; UAS.myrLgl-GFP/+*
Figure 4.8 D, J-K	*yw, tub.GAL4, hs.FLP/+ ; FRT40A lgl^{4}/FRT40A CD21 y+ GAL80; UAS.LglASA-GFP/+*
Figure 4.8 H	*lgl^{4}/lgl^{334} ; tub.GAL4/UAS.Lgl-GFP*
Figure 4.8 L	*lgl^{4}/lgl^{334} ; tub.GAL4/UAS.LglASA-GFP*
Figure 4.9 C	*w; wor.GAL4+ ; UAS.Lgl-GFP/+*
Figure 4.9 D, F-G	*w; wor.GAL4+ ; UAS.LglASA-GFP/+*
Figure 4.9 E	*w; wor.GAL4+ ; UAS.myrLgl-GFP/+*
Figure 4.10 A, E	*yw, tub.GAL4, hs.FLP, UAS.nucGFP-myc/+ ; FRT40A lgl^{4}/FRT40A CD21 y+ GAL80*
Figure 4.10 B, F	*yw, tub.GAL4, hs.FLP/+ ; FRT40A lgl^{4}/FRT40A CD21 y+ GAL80; UAS.Lgl-GFP/+*
Figure 4.10 C	*yw, tub.GAL4, hs.FLP/+ ; FRT40A lgl^{4}/FRT40A CD21 y+ GAL80; UAS.LglASA-GFP/+*
Figure 4.10 D, H	*yw, tub.GAL4, hs.FLP/+ ; FRT40A lgl^{4}/FRT40A CD21 y+ GAL80; UAS.myrLgl-GFP/+*
Figure 4.10 G	*yw, tub.GAL4, hs.FLP/UAS.LglASA ; FRT40A lgl^{4}/FRT40A CD21 y+ GAL80; +*

Figure 4.11 B	*yw, tub.GAL4, hs.FLP/+ ; FRT40A lgl^4/FRT40A CD21 y+ GAL80; UAS.Lgl-GFP/+*
Figure 4.11 C	*yw, tub.GAL4, hs.FLP/+ ; FRT40A lgl^4/FRT40A CD21 y+ GAL80; UAS.LglASA-GFP/+*
Figure 4.11 D	*yw, tub.GAL4, hs.FLP/+ ; FRT40A lgl^4/FRT40A CD21 y+ GAL80; UAS.myrLgl-GFP/+*
Figure 5.1 A-B	*ubi.Arm-GFP*
Figure 5.1 C-D	*E-Cad-GFP*
Figure 5.2 A-B	*sqh.sqh-GFP*
Figure 5.2 C	*E-Cad-GFP*
Figure 5.3 A-E	*sqh.sqh-GFP*
Figure 5.4 B	*lgl^{334}/lgl^{334}*
Figure 5.5 A	*yw, hs.FLP/+ ; FRT40A/FRT40A GFP*
Figure 5.5 B	*yw, hs.FLP/+ ; FRT42D $aPKC^{K06403}$/FRT4D GFP*
Figure 5.5 C	*yw, hs.FLP/+ ;; FRT82B $scrib^2$/FRT82B GFP*
Figure 5.5 D	*yw, hs.FLP/+ ; FRT40A lgl^4/FRT40A GFP*
Figure 5.5 E	*yw, hs.FLP/+ ; FRT42D shg^{R69}/FRT42D GFP*
Figure 5.5 F	*yw, hs.FLP, FRT19A baz^4/FRT19A GFP*
Figure 5.5 G	*yw, hs.FLP/+ ;; FRT82B crb^{82-04}/FRT82B GFP*
Figure 5.5 H	*yw, hs.FLP, dlg^{M52} FRT101/FRT101 GFP*
Figure 5.6 B	*ptc.GAL4, UAS.GFP/UAS.E2F, UAS.Dp*
Figure 5.6 C	*ptc.GAL4, UAS.GFP/UAS.E2F, UAS.Dp; UAS.p35/+*
Figure 5.6 D	*yw, Actin>CD2>GAL4, hs.FLP, UAS.nucGFP-myc/+; UAS.E2F, UAS.Dp/+ ; UAS.p35/+*
Figure 5.7 B	*MS1096.GAL4/+ ; UAS.Ras^{V12}/+*
Figure 5.7 C-G	*yw, Actin>CD2>GAL4, hs.FLP, UAS.nucGFP-myc/+; UAS.Ras^{V12}*
Figure A.2 A	*yw, tub.GAL4, hs.FLP/+ ; FRT42B $aPKC^{265}$/FRT42B CD21 y+ GAL80; UAS.Lgl-GFP/+*
Figure A.2 B	*yw, tub.GAL4, hs.FLP/+ ; FRT42B $aPKC^{K06403}$/FRT42B CD21 y+ GAL80; UAS.Lgl-GFP/+*
Figure A.4 A-B	*MS1096.GAL4/+ ;; UAS.AurA-GFP;+*

Figure A.5 B-D	*MS1096.GAL4/+ ;; UAS.myrLgl-GFP;+*
Figure A.5 E-G	*MS1096.GAL4/+ ;; UAS.Lgl-GFP;+*
Figure A.5 H-J	*MS1096.GAL4/+ ;; UAS.Lgl3A-GFP;+*
Figure A.5 K-N	*MS1096.GAL4/+ ;; UAS.LglASA-GFP;+*
Figure A.6 A-B	*yw, tub.GAL4, hs.FLP/+ ; FRT40A lgl^4/FRT40A CD21 y+ GAL80; UAS.myrLgl-GFP/+*
Figure A.6 D	*yw, tub.GAL4, hs.FLP/+ ; FRT40A lgl^4/FRT40A CD21 y+ GAL80; UAS.LglASA-GFP/+*
Figure A.7 A-B	*aurora A^{8839}/aurora A^{14641}*
Figure A.8 B-F	*ptc.GAL4, UAS.GFP/UAS.ECad7*
Figure A.9 A-C	*yw, Actin>CD2>GAL4, hs.FLP, UAS.nucGFP-myc/+; UAS.yki-nls*

Genotypes in movies are as follows:

Movie M1	As Figure 3.1 A-C
Movie M2	As Figure 3.2 A-C
Movie M3	As Figure 3.4 C
Movie M4	As Figure 3.5 A-B
Movie M5	Top left: *ECad-GFP* Top right: *ECad-GFP/+ ; aurora A^{87Ac-3}/Df(3R)Exel6161* Bottom left: *en.GAL4, ECad-GFP/UAS.Aurora B RNAi* Bottom right: *ECad-GFP*
Movie M6, M15	As Figure 3.12 A
Movie M7	As Figure 4.4 A
Movie M8, M9	As Figure 4.4 B
Movie M 10	As Figure 5.1 A-C
Movie M11	As Figure 5.2 A-B
Movie M12	As Figure 5.2 C
Movie M13	As Figure A.2 A
Movie M14	As Figure A.4 B
Movie M16	*ECad-GFP, ptc.GAL4,/UAS.E2F, UAS.Dp*
Movie M17	*en.GAL4, ECad-GFP/UAS.Aurora B RNAi*

2.2 Histology

2.2.1 Transmission electron microscopy of wing discs

Processing (steps iv-x) and imaging of the samples were carried out by Ken Blight from the Cancer Research UK Electron Microscopy Unit.

i. Dissect wing discs from third instar larvae in cold PBS containing 4% PFA
ii. Transfer to coverslips coated with poly-L-lysine
iii. Fix in 0.1M phosphate buffer pH 7.4, containing 4% PFA and 2.5% glutaraldeyde for 2 hours at room temperature
iv. Post-fix discs in 1% osmium/1.5% potassium ferrocyanide for 1 hour
v. Incubate coverslips in 1% tannic acid in 0.05M sodium cacodylate pH 7.4 for 45'
vi. Dehydrate coverslips stepwise through ethanol
vii. Incubate with 50:50 propylene oxide:epon followed by one change of purersin every 24 hours for 7 days
viii. Incubate at 60C overnight for polymerisation
ix. Collect ultrathin sections of ~75nm using a UCT ultramicrotome (Leica Microsystems UK),
x. Post-stain with lead citrate and viewed using a Tecnai G2 Spirit 120kV transmission electron microscope (FEI Company) with an SC1000 Orius CCD camera (Gatan UK).

2.2.2 Immunostaining of *Drosophila* tissues

Wing or eye imaginal discs were dissected from third instar larvae in ice cold PBS. The majority of irrelevant tissues were removed (fat body, gut etc.) and samples were processed as follows:

i. Fix for 30 minutes in cold PBS containing 4% paraformaldehyde
ii. Rinse once in PBT then wash and permeabilise for 10 mins in PBT
iii. Incubate samples in BBT and primary antibody for either 2 hours at room temperature, or overnight at 4C
iv. Wash 4x30 mins in BBT

v. Incubates samples in PBT and secondary antibody for 2 hours at room temperature
vi. Wash 3x10 mins in PBT. In the first wash, add 1 µg/ml DAPI (Molecular Probes) to stain DNA.
vii. Mount in Fluoromount G (Southern Biotechnology)

Ovaries were prepared and processed as follows:

i. The day before dissection, place adult females in a vial with some males and wet yeast.
ii. Dissect ovaries from females in ice cold PBS. Gently open ovaries to allow infiltration of solutions.
iii. Fix for 25 minutes in cold PBS containing 4% paraformaldehyde
iv. Rinse once in PBT then wash and permeabilise for 10 mins in PBT
v. Block for 30 mins in PBT containing 5% NGS (Normal Goat Serum)
vi. Incubate samples in PBT + 5% NGS and primary antibody either for 2 hours at room temperature, or overnight at 4C
vii. Wash 4x30 mins in BBT
viii. Incubates samples in PBT and secondary antibody for 2 hours at room temperature
ix. Wash 3x10 mins in PBT. In the first wash, add 1 µg/ml DAPI (Molecular Probes) to stain DNA.
x. Remove PBT and add 2 drops of Vectashield. Mount in Vectashield no more than a day before microscopy analysis.

Solutions used:

PBS (Phosphate buffered saline):
NaCL 8 g/L, KCl 0.25 g/L, Na_2HPO_4 1.43 g/L, KH_2HPO_4 0.25 g/L in distilled water.
PBT: PBS + 0.2% Triton X-100.
BBT: PBT + 0.1% BSA (Bovine Serum Albumin, Sigma)

Samples were imaged on a Leica SP5 confocal microscope and images processed in Adobe Photoshop and ImageJ. Quantifications were performed in ImageJ.

2.2.3 Western blotting

Actin5c.GAL4/UAS.Lgl-GFP or Actin5c.GAL4/UAS.Lgl3A-GFP third instar larvae were dissected in cold PBS, and homogenised in sample buffer (50mM Tris pH7.5, 150mM NaCL, 1% Triton X-100, 1μM EGTA) supplemented with protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail (Roche) where appropriate. Samples were incubated for 30 minutes: with 2000 units/ml λ protein phosphatase (New England Biolabs), with 2mM VX-680 or on ice.

SDS sample buffer and reducing agent (NuPage, Invitrogen) were added and samples heated at 70C for 10 minutes before SDS Page and Western Blotting (NuPage, Invitrogen). Samples were resolved on a 4-12% Bis-Tris precast polyacrylamide gradient gel (NuPAGE). Protein sizes were marked using the Rainbow molecular weight marker (Amersham).

Proteins were transferred to nitrocellulose membranes using the iBlot system (Invitrogen). Membranes were processed accordingly:

i. Incubate membranes for 1 hr in block solution (TBST + Milk for non-phospho antibodies, or TBST + BSA for phospho-antibodies)
ii. Incubate in block solution with the primary antibody for 2 hrs at room temperature
iii. Rinse 3 times with TBST, and then wash 3x5mins in TBST
iv. Incubate in blocking solution with secondary antibody for 2 hours at room temperature
v. Rinse 3 times with TBST, and then wash 3x5 mins in TBST
vi. Incubate with Super Signal West Pico Chemiluminescent Substrate (Pico) according to manufacturer's instructions

Stripping of blots was performed using the Millipore re-blot kit where necessary.

Solutions used:

TBS (Tris-buffered saline): NaCl 8g, KCl 0.2g, Tris base 3g, in 800ml distilled water. pH was adjusted to 8.0 using 1M HCl, and volume adjusted to 1000ml.

TBST: TBS + 0.1% Tween.

Blocking solutions: TBST + 5% dried milk power (for non-phospho antibodies) or TBST + 5% BSA (Sigma) (for phospho-antibodies).

2.3 Antibodies

For **immunofluorescence**:

Rabbit aPKC (Santa-Cruz) 1:250
Mouse phosphohistone H3 (Abcam) 1:1000
Rabbit phosphohistone H3 (Millipore) 1:1000
Rabbit phospho-LLGL1/2 (Abgent) 1:250
Rabbit Centrosomin (1:1000) (Lucas and Raff, 2007)
Mouse GFP (Roche) 1:250
Rabbit GFP (AMS Biotechnology) 1:250
Mouse α-Tubulin DM1A (Sigma) 1:500
Rabbit Lgl (Santa-Cruz) 1:250
Rabbit Cleaved Caspase-3 (Cell Signalling) 1:500
Rabbit γ-tubulin (Sigma) 1:500
Rabbit Pins (Yu et al., 2000) 1:1000
Mouse Dlg (DSHB) 1:250
Mouse Miranda (Ohshiro et al., 2000) 1:50
Rat DE-Cadherin (DSHB) 1:100
Rat ELAV (DSHB) 1:300
Rabbit phospho-Myosin Light Chain II (Cell Signalling) 1:50
Secondary antibodies (all from Molecular Probes, Invitrogen) were used at 1:500.

For **Western Blotting**:

Rabbit phospho-LLGL1/2 (Abgent) 1:100
Mouse α-Tubulin (DSHB) 1:1000
HRP-conjugated secondary antibodies (Thermo Scientific) 1:10,000

2.4 Live imaging

Live imaging of *ex vivo* cultured wing discs was performed largely as previously described (Aldaz et al., 2010). Third instar larvae were dissected, and wing imaginal discs were cultured in Shields and Sang M3 media (Sigma) containing 2% FBS (fetal bovine serum), 10µg/ml streptomycin/penicillin (Invitrogen), 10mU/L insulin (Sigma), 0.1 µg/ml ecdysone (Sigma) and 2.5% methyl cellulose (Sigma). Samples were imaged in a 35mm-Fluorodish (World Precision Instruments) on a Zeiss 780 confocal microscope, or a Perkin-Elmer Spinning Disc microscope. Z-stacks were taken typically at 1-µm intervals with a total thickness of 10-30µm. Z-stacks were typically scanned at 90s intervals for up to 3 hours. Images were projected and time points collated using either Zen or Volocity software.

For drug treatment, inverted larvae were incubated in the culture media as described above (without methyl cellulose) with 2mM VX-680/Tozasertib (Selleck BioChem), 2.5mM Y-27632 (Millipore) or 0.1 mg/ml Colchicine (Sigma) for 30-60 minutes, then transferred to media as described above for imaging. In some instances, imaging was performed in media containing the drug.

2.5 Quantifications and image analysis

Images were processed in Adobe Photoshop and analysed with ImageJ. For **cytoplasmic intensity quantifications** (Figure 3.2, 3.4, 3.7, 3.1 and A.1), image intensity was measured for a region in the cytoplasm of a mitotic cell, and compared to the image intensity for a region in the cytoplasm of interphase cells. Cortical:cytoplasmic ratios were not used in this analysis to prevent confusion as to which of two adjoining cells the cortical signal is coming from, though analysis of cells on the edge of a clone could overcome this. To account for variations in image quality between samples/movies, mitotic and interphase cells were compared directly only in the same image. Averages of these relative numbers were taken over multiple images. Verification was performed using multiple GFP-tagged lines and/or antibody stainings where possible. Anti-GFP antibody was sometimes used for GFP-tagged lines in the follicle cell epithelium to aid in analysis.

Errors bars are given as the standard deviation of the mean. Statistical analysis of mitotic vs. interphase cells was performed using a two-tailed t test. An asterisk denotes a p value < 0.05.

For analysis of junctional intensity (Figure 5.1), image intensity was measured along the junction of a mitotic cell (shared with its neighbouring interphase cell) and compared to the intensity of a junction of an interphase cell (shared with another interphase cell). Note that these 'mitotic cell' measurements therefore include the contribution from both the mitotic and the neighbouring cell. Quantification was not performed on two neighbouring cells both in mitosis, due to low frequencies of this event occurring. Mean junctional intensity was the measured intensity relative to the length of the junction; total junctional intensity is the total measurement around the mitotic cell. Statistical analysis of mitotic vs. interphase cells was performed using a two-tailed t test. An asterisk denotes a p value < 0.05.

For **spindle angle** analysis in wings or follicle cells, the spindle was measured by comparing the axis of tubulin staining with the plane of the epithelium (as marked by antibody staining). Mitotic cells with disorganised spindles, or rare cases of apparent tripolar spindles, were excluded from this analysis. Values were plotted in rank order from lowest to highest, with spindle angles on the x axis. In neuroblasts, spindle angle was measured as the deviation of the axis of the tubulin staining relative to the apical crescent (marked by antibody staining), and values were plotted in 15^{o} segments in a radial chart. Metaphase spindle angles in mutants or rescue experiments in wing discs or follicle cells were compared relative to wild type using the Kolmogorov-Smirnoff test. No statistical analysis was performed for spindle orientation in neuroblasts.

For **clone size** analysis, the percentage of a wing disc or ovariole comprising clonal tissue (positively marked by GFP using the MARCM system) was averaged over a number of discs or ovarioles. Error bars are given as standard deviation of the mean, and statistical analysis was performed using a two-tailed t test. An asterisk denotes a p value < 0.05. Projections of z stacks comprising the whole disc (wings) or encompassing the whole clone (ovaries) were used.

Centrosome numbers in ***lgl* or *dlg* mutant wing discs** were quantified using projections of large z stacks encompassing the mitotic cell in question (to guard against misoriented centrosomes/spindles in *lgl* or *dlg* mutant discs). 'Aberrant' spindles (in wing discs) were defined using tubulin staining as being mono- (or multi-) polar or generally disorganised compared to wild type. Projections of large z stacks and cross sections through mitotic cells were used to ascertain that spindles were defectively organised rather than simply misoriented. Nuclear defects were measured by examining DAPI staining in wt or *lgl* mutant discs and quantifying multi-nucleate cells or otherwise fragmented nuclei. Statistical analysis was performed using a two-tailed t test. An asterisk denotes a p value < 0.05.

2.6 *In vitro* kinase assay

In vitro kinase assays were performed using short peptides of potential substrates, which contained the kinase consensus sequence and putative phosphorylation sites. CENP-A was used as a positive control for both Aurora A and B. Results plotted show the mean of triplicate experiments, and error bars show the standard deviation of the mean. Kinase assays were performed as follows:

i. Make substrate solution of HPLC-purified peptides (in distilled water) and 5x kinase buffer (see below)
ii. Add 200ng Aurora A (Promega) or 80ng Aurora B (Promega) recombinant protein to the substrate solution
iii. Add 10μM cold ATP and 3μCi of [Y-P32] ATP (Perkin Elmer) to each sample, and incubate for 30' at 30C
iv. Pipette solution onto squares of P81 phosphocellulose paper (Millipore) and air dry for 2s
v. Wash squares 3x10 mins in 1% phosphoric acid
vi. Wash in acetone
vii. When dry, transfer squares to scintillation vials for counting by liquid scintillation (Beckman LS 6500)

Reaction Buffer (Promega):

200mM Tris-HCL (pH 7.5), 100mM $MgCl_2$, 0.5 µg/µl BSA, 50µM DTT

2.6.1 Peptides used in kinase assay

Peptides were generated by the London Research Institute Peptide Synthesis lab, and were:

Lgl	LSRRKSFKKSLRESFRKLR (2422.91 Da)
Lgl3A (S556A, S660A, S664A)	LSRRKAFKKALREAFRKLR (2374.91 Da)
LglASA (S656A, S664A)	LSRRKAFKKSLREAFRKLR (2390.91 Da)
LglSAS (S660A)	LSRRKSFKKALRESFRKLR (2406.91 Da)
LglAAS (S656A, S660A)	LSRRKAFKKALRESFRKLR (2390.91 Da)
LglASS (S656A)	LSRRKAFKKSLRESFRKLR (2406.91 Da)
CENP-A	MGPRRRSRKPEAPRRRSPSP (2374.77 Da)
CENP-A (S7A)	MGPRRRARKPEAPRRRSPSP (2358.77 Da)
Sqh/MRLC	TTKKRAQRATSNVFAMFDQA (2271.59 Da)
Sqh/MRLC AA (T18A, S19A)	TTKKRAQRAANVFAMFDQA (2225.56 Da)

Peptides were diluted with deionised water to working dilutions (1mg/ml) and stored at -20°C.

2.7 Molecular biology: construct generation

Transgenic flies were generated using the Quikchange mutagenesis kit to alter DNA and the Gateway recombination cloning system to create UAS.X(-GFP) constructs.

2.7.1 Generation of AurA constitutively-active constructs

Aurora A is phosphorylated in an activation-loop at Thr295 in *Drosophila* (Thr288 in Humans) to aid its activity. We attempted to generate phospho-mimetic constructs of Aurora A with this site mutated to Aspartic Acid (D) or Glutamic Acid (E).

2.7.1.1 cDNA

Aurora A cDNA was obtained from clone LD16949 from the *Drosophila* Genomics Resources Centre (DGRC), and processed according to instructions. Details below:

i. Add 50µl of sterile TE to tube containing the clone disc, and pipette up and down. Quickly remove TE, and place tube on ice
ii. Add 50µl of Top10a competent cells and incubate on ice for 30 mins. Vortex halfway through
iii. Heat shock tube for 30 seconds at 42C
iv. Transfer cells to 1ml of SOC media and incubate with shaking at 37C for 1 hour
v. Plate 100µL on LB plates with Chloramphenicol. Incubate overnight at 37C
vi. Pick colonies and perform miniprep using QIAprep Spin Miniprep kit, in accordance with manufacturer's instructions

2.7.1.2 Mutagenesis

Primers were designed in accordance to Quikchange Site-Directed Mutagenesis Kit recommendations. Primers to generate Aurora mutants $AurA^{T295E}$ and $AurA^{T295D}$ were as follows:

Wild type sequence of Aurora A with Thr295 highlighted:
5'…AACTCCATGCGCATG***ACA***CTGTGCGGCACTGTC…3'

Primers for mutation to E:
Forward: 5' AACTCCATGCGCATG***GAG***CTGTGCGGCACTGTC 3'
Reverse: 5' GACAGTGCCGCACAG***CTC***CATGCGCATGGAGTTCGG 3'

Primers for Mutation to D:
Forward: 5' AACTCCATGCGCATG***GAC***CTGTGCGGCACTGTC 3'
Reverse: 5' GACAGTGCCGCACAG***GTC***CATGCGCATGGAGTTCGG 3'

PCR mutagenesis reactions were performed as follows, using 10ng template DNA (from Aurora A clone).

PCR conditions:

i. 95C for 30s
ii. 95C for 30s
iii. 55C for 60s
iv. 68C for 5 mins
v. Repeat ii-iv x 15

Mutagenesis procedure continued according to the Quikchange protocol: transforming Top10a cells with the PCR reaction - incorporating a 42C heat shock for 30s - and plating on Ampicillin plates for overnight incubation and miniprepping as standard.

Sequencing primers to check the mutagenesis were as follows:
Aur Seq 1: 5' CTGTATGCCTACTTTCACGACGACG 3'
Aur Seq 2: 5' GAGCGGGACATCATACACAGGGACATC 3'

2.7.1.3 Gateway cloning

Sequencing confirmed correct mutagenesis. The sequenced constructs were then used as template for Gateway cloning, using the following attB primers:

5' GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGTCCCATCCGTCTGACC ATGTGC 3'
5' GGGGACCACTTTGTACAAGAAAGCTGGGTCCTGCGTGTGTCGCCAGGA 3'

attB PCR products were cloned into Gateway entry vectors using BP recombinase. Entry vectors were then transferred into expression vectors by LR recombination according to manufacturer's instructions. Expression vector pPW was used to generate UAS.AurA$^{T295D/E}$ constructs.

2.7.2 Generation of LglASA constructs

From our kinase assays, we determined that Aurora A phosphorylated Lgl on S656 and S664. aPKC can also phosphorylate these residues, and additionally S660. An $Lgl^{S656A,\ S664A}$ construct, designated LglASA, should therefore be insensitive to phosphorylation by Aurora A. We generated this construct similarly to the Aurora constructs, using Quikchange mutagenesis and Gateway cloning.

2.7.2.1 cDNA

Lgl cDNA was obtained from clone LD06034 from the *Drosophila* Genomics Resources Centre (DGRC), and processed according to instructions.

2.7.2.2 Mutagenesis

Primers were designed in accordance to Quikchange Site-Directed Mutagenesis Kit recommendations. Mutagenesis of S656 and S664 was performed with 2 step-wise mutagenesis reactions: of either S656A first and S664A second, or vice versa.

Primers to generate Lgl mutants were:

Lgl sequence showing S656, S664:
5'…GAGAGCAGCTGTCTCGTCGAAAG***TCT***TTTAAGAAATCATTGAGGGAG***TCA***
TTTAGAAAGCTTCGC…3'

Primers for S656 mutation to A:
Forward: 5' CTGTCTCGTCGAAAG***GCA***TTTAAGAAATCATTG 3'
Reverse: 5' CAATGATTTCTTAAA***TGC***CTTTCGACGAGACAG 3'

Primers for S664 mutation to A:
Forward: 5' TCATTGAGGGAG***GCA***TTTAGAAAGCTTCGC 3'
Reverse: 5' GCGAAGCTTTCTAAA***TGC***CTCCCTCAATGA 3'

PCR mutagenesis reactions were performed as follows, using 10ng template DNA (from Lgl clone).

PCR conditions:

i. 95C for 30s
ii. 95C for 30s
iii. 55C for 60s
iv. 68C for 8 mins
v. Repeat ii-iv x 15

Mutagenesis procedure continued according to the Quikchange protocol: transforming Top10a cells with the PCR reaction - incorporating a 42C heat shock for 30s - and plating on Ampicillin plates for overnight incubation and miniprep as standard.

Sequencing primers to check the mutagenesis were as follows:

Lgl Seq 1: 5' GTTATAGCCGACTTCATAGACTTACC 3'
Lgl Seq 2: 5' GATCGTGATGGATTTGTGTGGAAGG 3'

2.7.2.3 Gateway cloning

Sequencing confirmed correct mutagenesis. The sequenced constructs were then used as template for Gateway cloning, using the following attB primers:

5' GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGTTAAAGTTTATCAGAGG 3'
5' GGGGACCACTTTGTACAAGAAAGCTGGGTCAAATTGGCTTTCTTCAG 3'

attB PCR products were cloned into Gateway entry vectors using BP recombinase. Entry vectors were then transferred into expression vectors by LR recombination according to manufacturer's instructions. Expression vectors pPW or pPGW were used to generate UAS.Lgl^{ASA} and UAS.GFP-Lgl^{ASA} constructs respectively. In the text, these are referred to as (UAS.)LglASA(-GFP). Transgenesis was performed by BestGene.

Chapter 3. Mitotic regulation of Lgl

3.1 Polarity determinants are maintained during mitosis, with the exception of Lgl, which relocalises to the cytoplasm

The initial characterisation of polarity during mitosis was carried out by live imaging fluorescently-tagged polarity determinants in the third instar larval wing imaginal disc, and observing any changes to localisation. In addition, live imaging of secondary fluorescently-tagged lines, and antibody staining in fixed tissues was used to confirm any results. We found that the majority of polarity determinants remain unchanged during mitosis (Figure 3.1 and Movies M1 and M2). In the wing disc, the apical proteins aPKC, Par-6 and Crumbs remain localised at the apical cortex of the cell: we observed no real spreading of these determinants laterally in mitotic cells in cross-sections (Figure 3.1 A-B, D-G) or sequential slices through z-stacks (data not shown): however detailed quantifications were not performed. Some accumulation of Crumbs-GFP appeared to be present as the cleavage furrow formed, but this was somewhat variable and the observation was not followed-up. We found that the adherens junctions components E-Cadherin and Armadillo (β-catenin) were down regulated during mitosis, but remained at a similar position to interphase cells (Figure 3.1 H and Chapter 5). Consistent with its slightly unclear localisation and function, Bazooka/Par-3 was found to be somewhat down regulated, but remains largely apical (Figure 3.1 C). The basolateral polarity determinants Dlg and Scribble remain localised at the basolateral membrane in their interphase positions (Figure 3.2 A-B). In contrast, Lgl, the third member of the Scribble module, relocalises from the cell cortex into the cytoplasm in mitosis (Figure 3.2 C, G). Note that whilst Dlg and Scribble are enriched at the septate junctions (Figure 3.1 G-H and data not shown), Lgl is localised more uniformly around the basolateral membrane (Figure 3.2 E). Examination of mitotic cells on the edge of a clone expressing Lgl-GFP revealed a complete relocalisation, with no signal remaining at the membrane (Figure 3.2 D-E). Subsequent to mitosis, Lgl is again found at the cell cortex, and not in the cytoplasm. Similar Lgl relocalisation to the cytoplasm was found in mitotic cells in the follicle cell epithelium (Figure 3.2 F, G). Quantification comprised a ratio of the cytoplasmic signal intensity of mitotic cells and cytoplasmic signal intensity of interphase cells. Quantification of the various polarity determinants is found in Figure A.1.

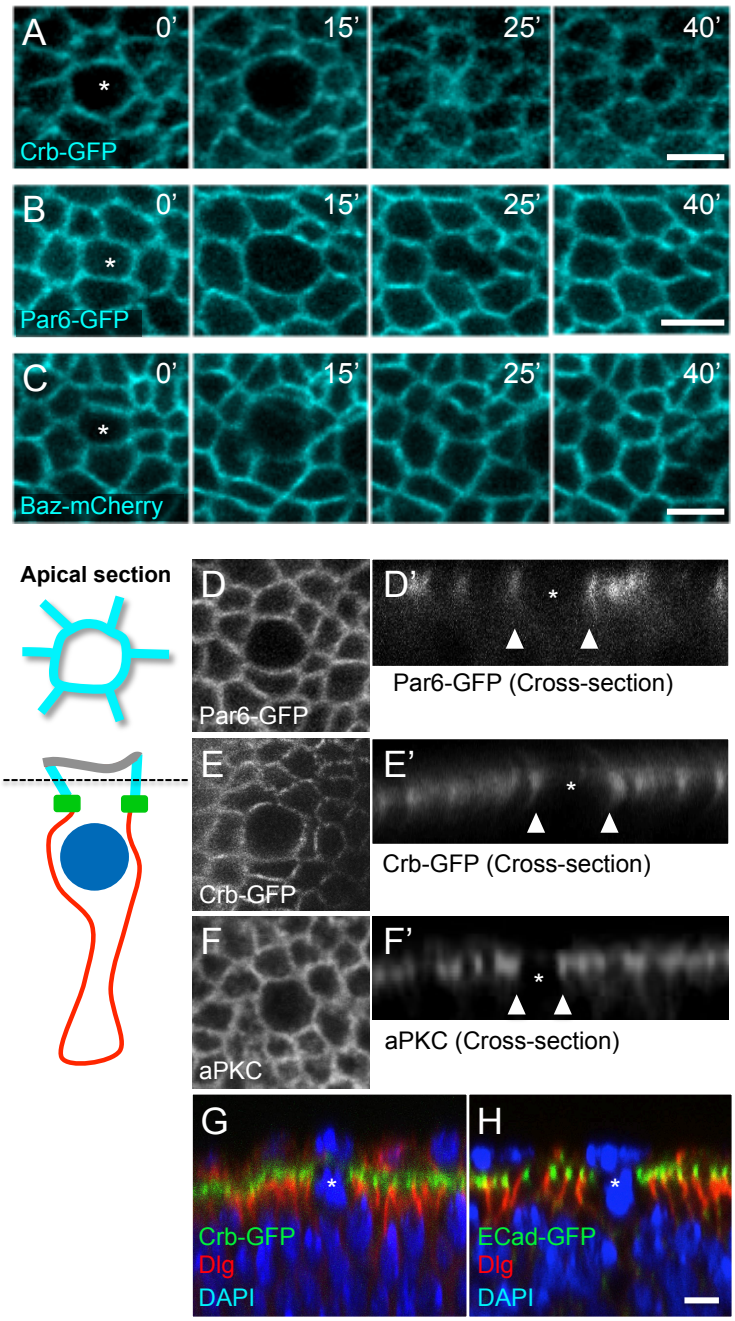

Figure 3.1 Apical polarity proteins are unchanged in mitosis in wing disc epithelia

(A-B) Fluorescently-tagged apical polarity determinants Crumbs and Par-6 remain apically localised during mitosis, as revealed by live imaging.
C) Fluorescently-tagged Bazooka/Par-3 is partially down regulated but remains apical.
(D-F) Top-down and cross-section views of Par6-GFP, Crb-GFP and aPKC antibody (diagrammed left). Note that in the cross-sections, apical determinants in mitotic cells remain at the same level as interphase cells (arrowheads indicate polarity determinants in mitotic cells, asterisks indicate mitotic cells).
(G-H) Cross-section views through wing discs expressing Crb-GFP (G) and ECad-GFP (H). Neither Crumbs nor ECad change localisation in mitotic cells.
Scale bars 5μm. See Movie M1.

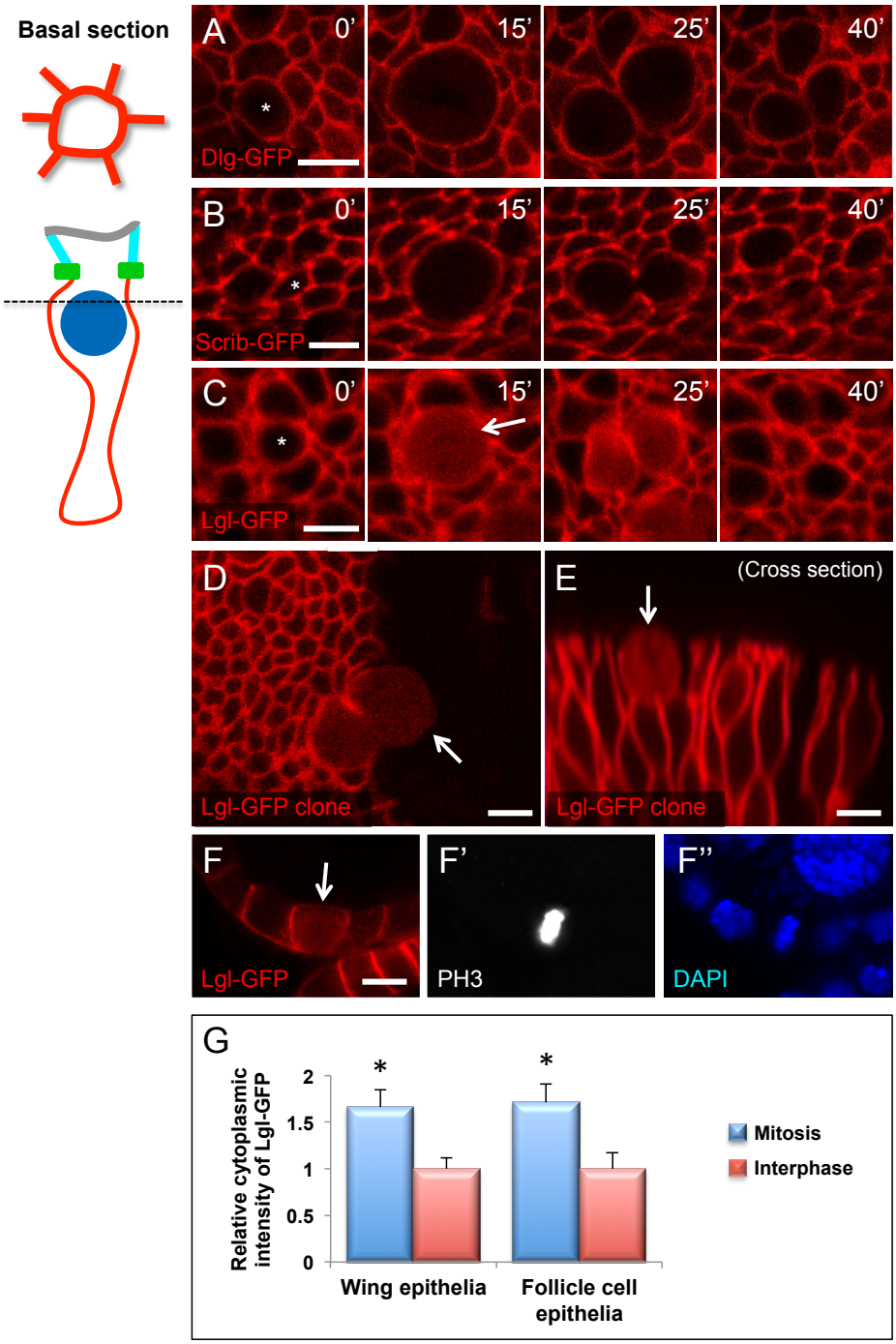

Figure 3.2 Lgl becomes cytoplasmic during mitosis in epithelial cells

(A-B) Fluorescently-tagged basolateral polarity determinants Dlg and Scribble remain cortical and enriched at the septate junctions during mitosis in wing discs (also see Figure 3.1).
(C-F) Fluorescently-tagged Lgl becomes cytoplasmic at mitosis. Note the complete relocalisation at mitosis with no membrane staining (D, arrow). Lgl-GFP is localised basolaterally in interphase (diagrammed left, and E. In E, the arrow denotes a mitotic cell). Lgl-GFP is present at the membrane again in daughter cells after mitosis (40' in C). (F) Lgl-GFP also relocalises to the cytoplasm in mitotic follicle cell epithelia. Quantification in G (mean ± SD, n≥10 for each sample). Quantifications of all polarity determinants are found in Figure A.1. Statistical analysis of mitotic vs. interphase cells was performed using a two-tailed t test. An asterisk denotes a p value < 0.05. Scale bars 5μm. See Movie M2.

3.2 Phosphorylation of Lgl regulates its mitotic localisation

Previous work has identified aPKC as a regulator of Lgl localisation by directly phosphorylating Lgl on a tripartite serine motif, comprising S656, S660 and S664 (*Drosophila* residues) (Betschinger et al., 2003; Wirtz-Peitz et al., 2008). These phosphorylation events are suggested to induce a conformational change in Lgl (Betschinger et al., 2005) resulting in an auto-inhibition of Lgl's ability to bind to the plasma membrane, and hence its removal from the cell cortex upon phosphorylation. Consistent with this, a phospho-Lgl antibody raised to detect phosphorylation of S656 and S660 strongly stains the cytoplasm of mitotic cells in both wing disc and follicle cell epithelia (Figure 3.3 A-B). This (cytoplasmic) signal is no longer present in mitotic lgl^4 mutant cells (Figure 3.3 C). Furthermore, use of the phospho-Lgl antibody in Western blotting of third instar larvae reveals a band of the correct size for Lgl only when expressing the wild type form of Lgl; but not Lgl3A, a non-phosphorylatable construct (Figure 3.3 D). The Lgl3A-GFP construct, which is unable to be phosphorylated by aPKC, is present apically in epithelial cells (Figure 3.4 B). In mitosis, Lgl3A-GFP does not relocalise to the cytoplasm in either wing disc or follicle cell epithelia (Figure 3.4 E and Movie M4). Thus, mitotic relocalisation of Lgl is a phosphorylation-dependent event involving the tripartite serine motif previously described.

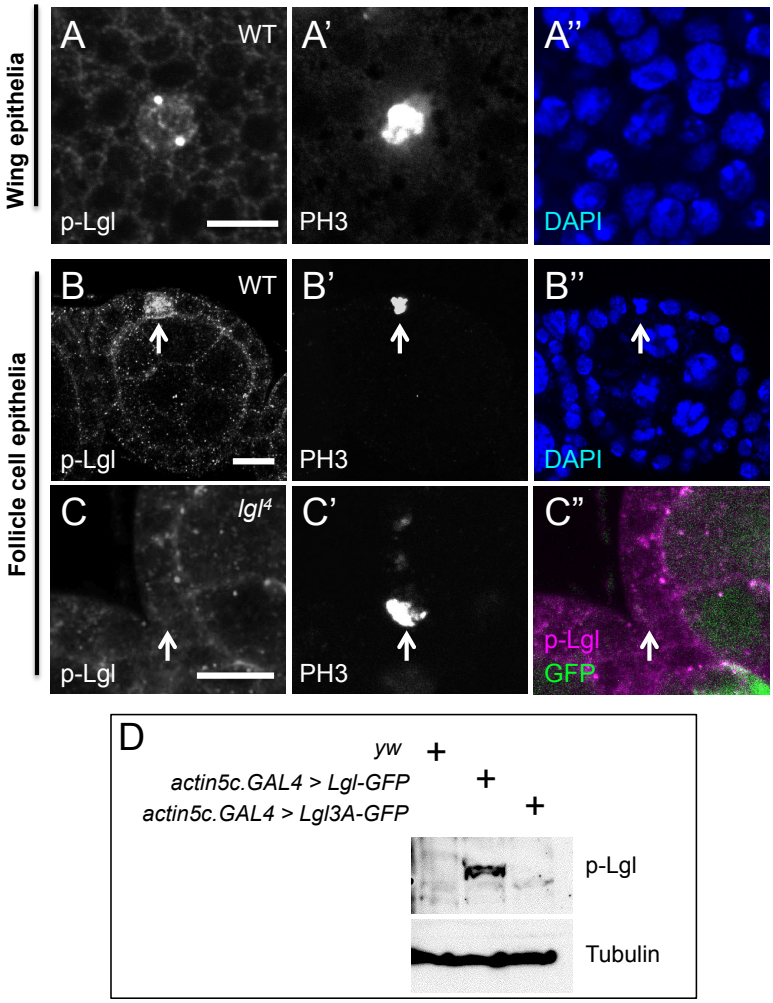

Figure 3.3 A phospho-Lgl antibody stains mitotic cells

(A-B) A phospho-Lgl antibody stains mitotic cells in wing disc (A) or follicle cell epithelia (B), with enrichment at the centrosomes (see Figure A.4).
(C) Staining of p-Lgl in mitotic cells is abolished in *lgl*^{4} mutant clones (marked by absence of GFP).
(D) The phospho-Lgl antibody is detectable by Western blotting analysis of third instar larvae expressing wild type Lgl, but not non-phosphorylatable Lgl3A.
Scale bars 10μm.

3.3 aPKC is not responsible for Lgl relocalisation in mitosis in epithelia

3.3.1 The role of aPKC kinase activity

Much of the work on Lgl regulation has been done studying asymmetrically dividing cells like neuroblasts. In this system, aPKC forms an apical crescent in mitotic cells, and phosphorylates cell fate determinants (like Miranda and Numb), restricting them to the opposite, basal side of the cell. Upon cell division, the daughter cells thus inherit unique cell fate determinants and acquire different fates. In contrast to this system, epithelial cells in the wing disc and ovary divide symmetrically, resulting in two identical daughter cells. The activation of aPKC in mitosis – and its formation of an apical crescent - is suggested as a key step for the subsequent events resulting in asymmetric division (Wirtz-Peitz et al., 2008). However, in epithelial cells, aPKC is constitutively active at the apical domain and maintains cell polarity in interphase. Furthermore, we do not observe aPKC altering its localisation in mitosis, such as spreading laterally. Thus a similar model to neuroblasts, where aPKC phosphorylates Lgl in mitosis to relocalise it away from the plasma membrane does not necessarily appear coherent.

Recently, the contribution of aPKC to epithelial polarity was investigated by use of *kinase-dead* mutant alleles (Kim et al., 2009). These mutants still permit the binding of aPKC to Par-6 and the formation of the apical polarity complex(es), and dissect the role of aPKC's kinase activity specifically from any other functions. Surprisingly, phenotypes of clones of *aPKC kinase-dead* were found to be quite mild (Kim et al., 2009). Given the theoretical differences in mitotic regulation of Lgl between epithelia and neuroblasts, and the new information on the importance of aPKC kinase activity, we used these constructs to ask whether the phosphorylation-dependent relocalisation of Lgl in mitosis is due to aPKC kinase activity.

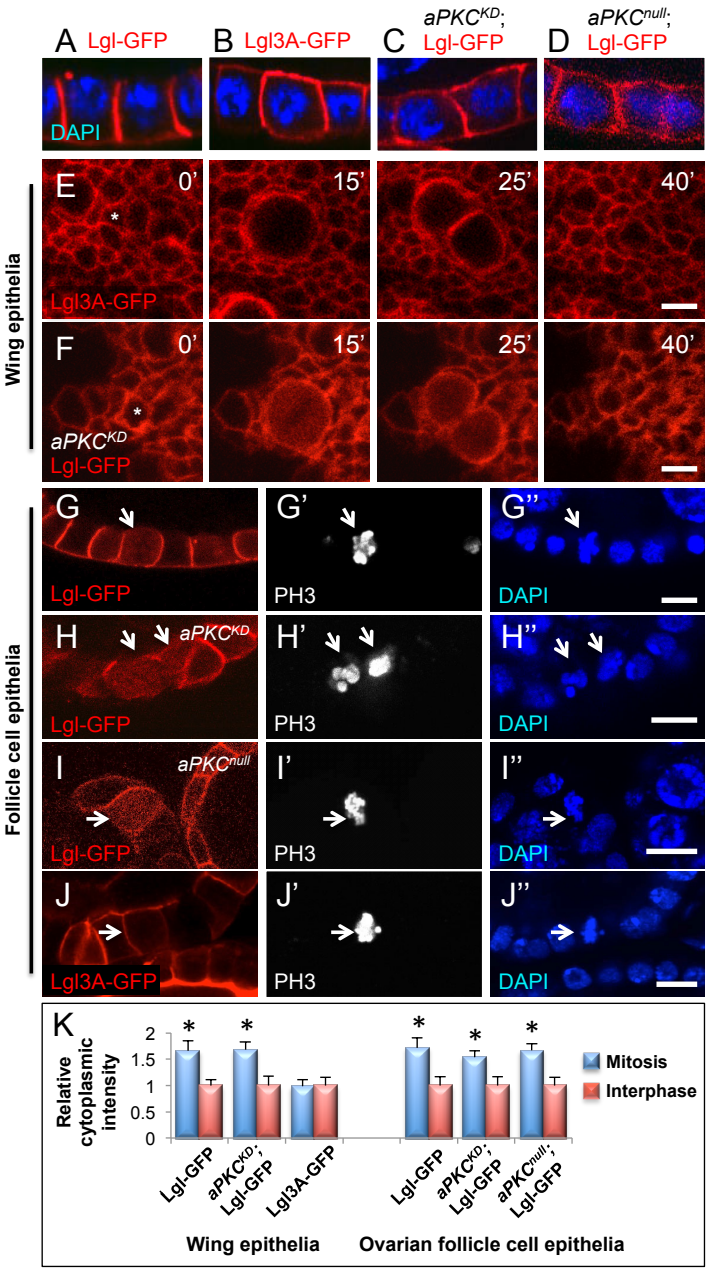

Figure 3.4 Lgl mitotic relocalisation is phosphorylation-dependent, but aPKC independent

(A-D) Phosphorylation of Lgl by aPKC is required to remove Lgl from the apical membrane. Lgl-GFP is normally restricted basolaterally (A). Non-phosphorylatable Lgl3A (B), or Lgl-GFP in *aPKC kinase-dead* (C) or *aPKC null* (D) mutant clones is present at the apical surface.
(E-F) Phospho-mutant Lgl3A-GFP fails to relocalise to the cytoplasm during mitosis in wing disc epithelia (E), but Lgl-GFP does relocalise normally in *aPKC kinase-dead* mutant clones (F).
(G-K) Lgl-GFP relocalises to the cytoplasm in mitotic ovarian follicle cell epithelia. This relocalisation stills occurs in *aPKC kinase-dead* (H) or *aPKC null* (I) mutant clones. Phospho-mutant Lgl3A-GFP fails to relocalise (J). The kinase-dead allele $aPKC^{psu417}$ was for this figure, but two other alleles ($aPKC^{psu265}$ and $aPKC^{psu141}$) showed identical results. Quantification in K (mean ± SD, n≥10 for each sample). Statistical analysis of mitotic vs. interphase cells was performed using a two-tailed t test. An asterisk denotes a p value < 0.05. Scale bars 5μm. See Movies M3 and M4.

3.3.2 Lgl relocalisation occurs in aPKC kinase-dead or aPKC null clones

We found that Lgl-GFP spreads apically in clones of either *aPKC null* or *aPKC kinase-dead* cells in the follicle cell epithelium, consistent with aPKC's role in removing Lgl from the apical membrane (Figure 3.4 A-D). Interestingly, we still observe the mitotic relocalisation of Lgl in either *aPKC kinase-dead* or *aPKC null* clones (Figure 3.4 H-I, Movie M3 and M13). These results were observed using any of the three available *kinase-dead* alleles of aPKC.

In the wing disc, we found that clones of *aPKC kinase-dead* cells (expressing Lgl-GFP) can be recovered easily – in contrast to *aPKC null* clones - consistent with the mild phenotype proposed in imaginal discs (Figure 3.4 F) (Kim et al., 2009). This was true for all three alleles of *aPKC kinase-dead* mutants. Similar to follicle cells, we found that relocalisation of Lgl still occurred in *aPKC kinase-dead* clonal cells (Figure 3.4 F and Movie M3). Mitotic cells on the edge of a clone also show complete absence of Lgl at the cortex (Figure A.2 and Movie M13). We also found that the accumulation of the phospho-Lgl antibody still occurs in mitotic cells in *aPKC kinase-dead* and *aPKC null* mutant clones (Figure 3.5 and Figure A.2).

Previously, it was suggested that expression of membrane-bound aPKC can force Lgl into the cytoplasm (Grifoni et al., 2007). We reproduced this experiment. However, in our hands, the expression of cortical aPKC did not perturb Lgl localisation (Figure 3.6). It may be that aPKC kinase activity is sufficient to remove Lgl from the apical membrane only in the presence of the other apical components. Par-6, for instance, has been reported as being able to activate aPKC (Graybill et al., 2012). Regardless, this result is consistent with our data that aPKC does not control Lgl mitotic relocalisation.

We conclude that whilst aPKC kinase activity is required for polarisation of Lgl in epithelia, the mitotic relocalisation is aPKC-independent, and thus mediated by a different kinase. Expression of cortical aPKC does not promote removal of Lgl from the membrane

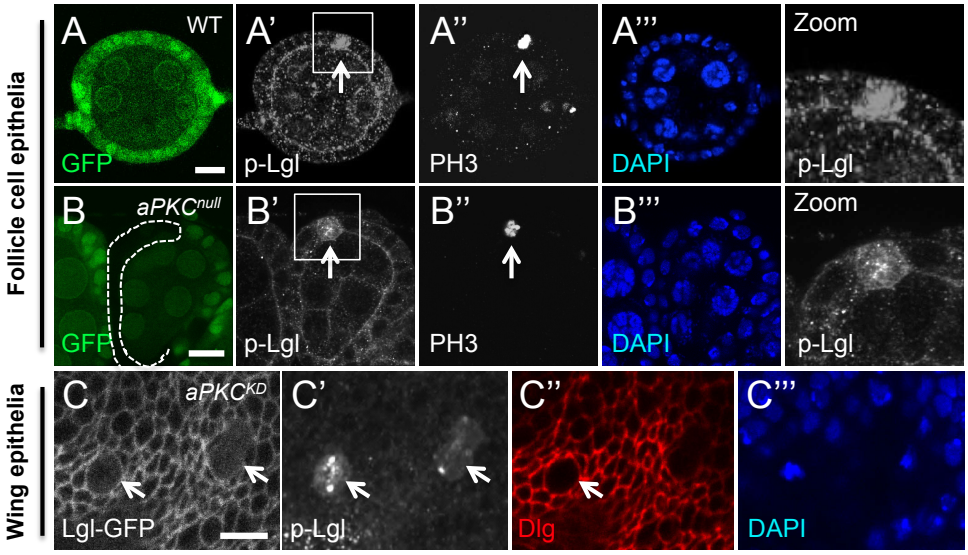

Figure 3.5 Phospho-Lgl mitotic accumulation is aPKC-independent

(A) In wild type follicle cells, phospho-Lgl antibody strongly stains mitotic cells (see also Figure 3.3).
(B) Clones of *aPKC null* mutant cells (marked by the absence of GFP) still show accumulation of phospho-Lgl in mitotic cells in follicle cell epithelia.
(C) Lgl-GFP and phospho-Lgl antibody are present in the cytoplasm of clones of mitotic *aPKC kinase-dead* cells in wing disc epithelia. Clones are marked by the presence of Lgl-GFP, which still relocalises to the cytoplasm (C, arrows). Note that Dlg is absent from the cytoplasm of mitotic cells (C'').
In (C), images shown are from experiments with $aPKC^{psu417}$ and $aPKC^{psu265}$ respectively, but the same phenotypes were observed with all 3 *kinase-dead* alleles of aPKC. Scale bars 10μm.

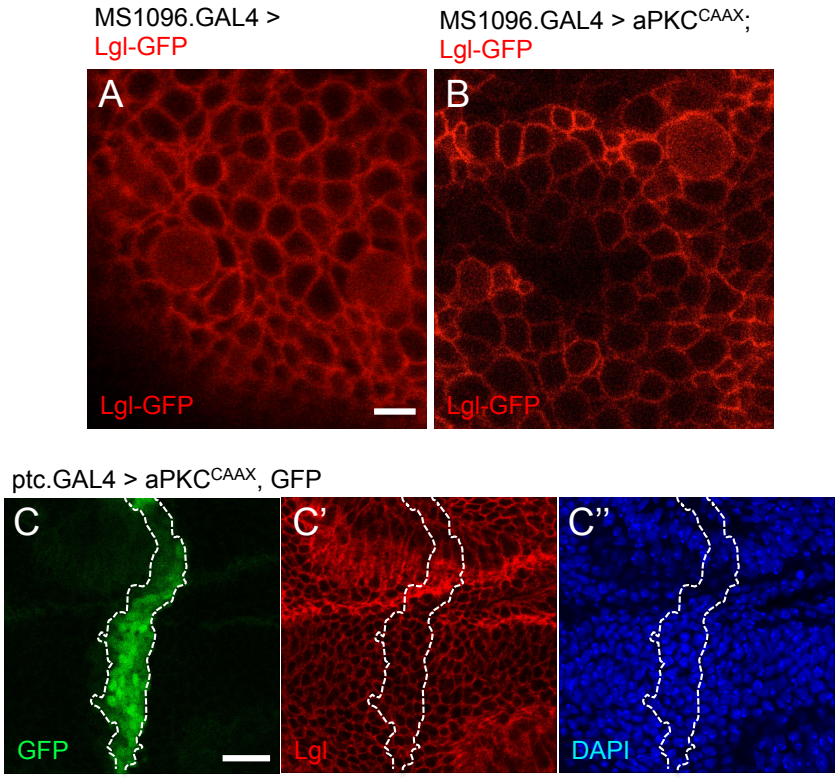

Figure 3.6 Expression of cortical aPKC does not induce Lgl relocalisation

(A-B) Localisation of Lgl-GFP is similar in wild type and aPKC^{CAAX}-expressing wing discs, with no ectopic Lgl relocalisation in aPKC^{CAAX} discs.
(C) Expression of aPKC^{CAAX} under the ptc.GAL4 promoter does not affect Lgl localisation, as shown by antibody staining, or perturb tissue integrity. Scale bars 5μm (A-B), 20μm (C).

3.4 Aurora kinases regulate Lgl localisation in mitosis

3.4.1 Speculation of the kinase responsible

Given that aPKC is not responsible for the mitotic relocalisation of Lgl, we next wondered which kinase(s) could be involved. The timing of the phenomena suggests the involvement of a cell cycle kinase, though not necessarily directly. An obvious candidate that had already been implicated in neuroblasts was Aurora A. Aurora A was previously proposed to phosphorylate Par-6, thus indirectly activating aPKC and resulting in the phosphorylation of Lgl (by aPKC) (Wirtz-Peitz et al., 2008). The consensus sequence of Aurora A matches two of the three serines in the Lgl tripartite motif that is required for Lgl mitotic localisation: S656 and S664, but not S660 (Carmena et al., 2009). Aurora A also has a similar localisation in mitosis to the staining of the phospho-Lgl antibody ((Berdnik and Knoblich, 2002), Figure A.4 and Movie M14). We therefore wondered whether Aurora A might be the kinase that phosphorylates Lgl and regulates its localisation in mitosis.

In their 2008 paper, Wirtz-Peitz et al had looked for any direct phosphorylation of Lgl by Aurora A, using a phospho-Lgl antibody in an *in vitro* kinase assay (Wirtz-Peitz et al., 2008). They could not see any band of phospho-Lgl using an Lgl peptide in the presence of Aurora A kinase, and therefore concluded that Aurora could not directly phosphorylate Lgl. However, the phospho-Lgl antibody that was used recognises only S660, the middle of the three serines, which does not match the Aurora A consensus sequence. We therefore continued to investigate whether Aurora A is in fact responsible for phosphorylating and regulating Lgl.

3.4.2 Lgl-GFP relocalisation in Aurora treated discs is delayed

We first examined the localisation of Lgl-GFP in *aurora A* mutant wing discs. *aurora A* mutant discs are viable to third instar larval stage, with normal epithelial structure and polarity, but display an increased mitotic index (Glover et al., 1995). We found that relocalisation of Lgl is strongly delayed, but does still eventually occur (Figure 3.7 B and Movie M4).

Two members of the Aurora family are expressed in *Drosophila*: Aurora A and B. Whilst Aurora A was previously implicated in Lgl regulation, and perhaps shows the localisation more plausible for interacting with Lgl, the two forms do share similar consensus sequences and have been reported to phosphorylate the same targets (e.g. CENP-A) (Kunitoku et al., 2003; Zeitlin et al., 2001). We therefore wondered whether there might be a redundancy between the two Auroras, and that removal of the both of them would fully prevent Lgl's relocalisation. We made use of the drug VX-680/Tozasertib, which is a dual Aurora A and B inhibitor (Huang et al., 2008). Treatment of wing discs with this drug results in few mitotic cells (see Section 3.5), and those mitotic cells present did not show relocalisation of Lgl-GFP into the cytoplasm, consistent with the idea of redundancy (Figure 3.7 C-E and Movie M4). We found also that phospho-Lgl staining in abolished in Western blotting upon treatment of VX-680 (or λ-phosphatase) (Figure 3.7 F).

One concern was that inhibiting both Auroras simply led to prevention of mitosis, and that those few escapers we observed happened to be caught in the particular time point(s) before Lgl would eventually relocalise. By altering culturing conditions and imaging large numbers of fixed discs, we were able to recover a number of cells that appeared to be mitotic, although PH3 staining could not be used as a marker, since it is dependent on Aurora B activity. In VX-680 treated discs, in cells that had undergone rounding and interkinetic nuclear migration – hallmarks of mitotic cells (Meyer et al., 2011; Stewart et al., 2011; Thery and Bornens, 2008) – we observed that on average, the cytoplasmic intensity of Lgl was comparable to interphase cells. In contrast, the rounded cells in fixed, untreated discs showed cytoplasmic intensity far higher than interphase cells Figure 3.7 G). Thus, we do not think that the absence of Lgl relocalisation in VX-680 treated discs is a mere by-product of imaging cells at precisely the point after cell rounding but before onset of relocalisation

These results suggest that the Auroras control Lgl relocalisation, but not necessarily directly. Although we had ruled out aPKC as the key kinase, it was possible that some other unidentified kinase was the key regulator of Lgl mitotic relocalisation. We therefore performed kinase assays to test whether the Auroras directly phosphorylate Lgl.

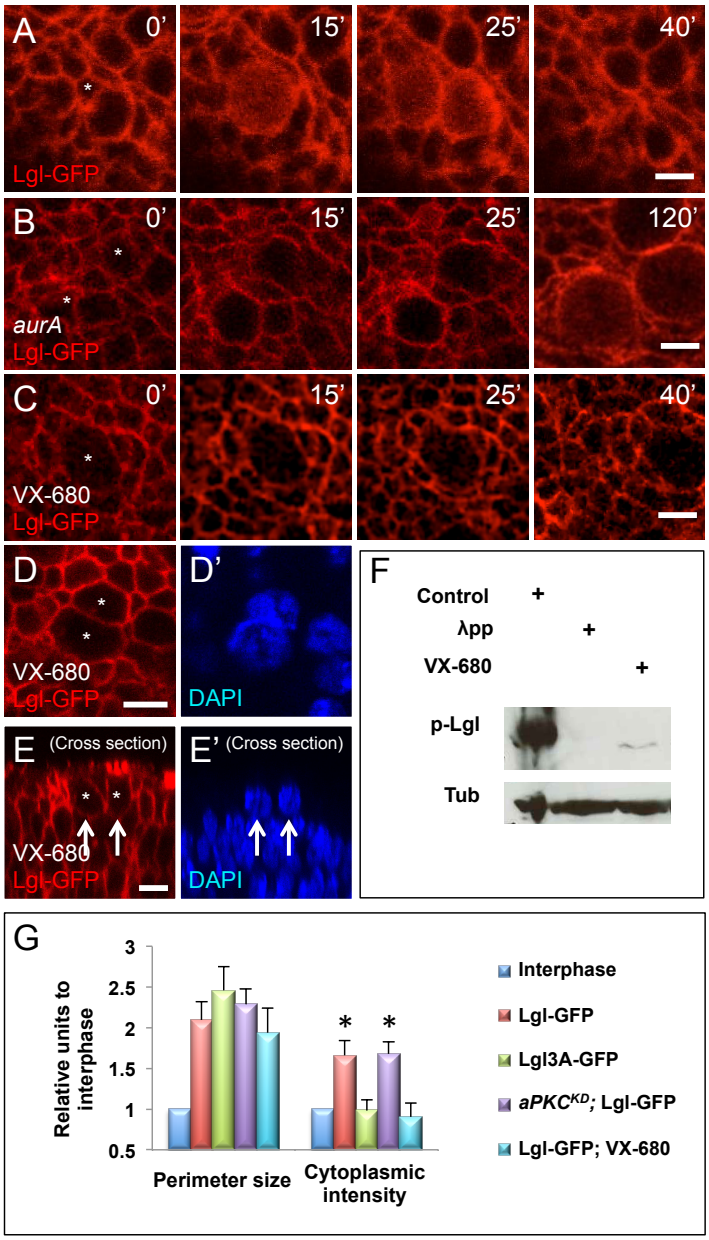

Figure 3.7 Aurora kinases control Lgl mitotic relocalisation

(A-E) Relocalisation of Lgl-GFP to the cytoplasm in mitosis is strongly delayed in *aurora A* mutants (B), and blocked upon treatment of the epithelium with the Aurora A/B inhibitor VX-680 (C-D). In VX-680 treated discs, few cells enter mitosis, but identification if mitotic cells is possible by examining cell rounding and interkinetic nuclear migration (D-E).
(F) A phospho-Lgl antibody shows a band in control Western blotting analysis, but not upon treatment with either VX-680 or λ-phosphatase. (All samples were actin5c.GAL4> Lgl-GFP third instar larvae, as in Figure 3.3).
(G) Validation of VX-680 drug treatment (mean ± SD, for perimeter size n=20 for each sample). Although few cells enter mitosis in drug-treated discs, cell rounding still occurs in escapers. Lgl-GFP in wild type or *aPKC kinase-dead* mitotic cells is enriched in the cytoplasm relative to interphase cells. Although Lgl3A-GFP and VX-680-treated mitotic cells still round up, relocalisation does not occur. Different combinations of *aurora* mutations showed similar results to those shown in (B). Perimeter size was measured at maximum size of cells through mitosis, presumably at metaphase. Statistical analysis of mitotic vs. interphase cells was performed using a two-tailed t test. An asterisk denotes a p value < 0.05. Scale bars 5μm. See Movie M4.

3.4.3 Aurora A and B directly phosphorylate Lgl *in vitro*

The kinase assays were carried out using peptides of Lgl encompassing the tripartite serine motif: S656, S660 and S664. Combinations of the various phospho-mutants of this region were generated: e.g. LglSSS ($Lgl^{S656, S660, S664}$) which is wild type; LglAAA ($Lgl^{S656A, S660A, S664A}$) which should be not be phosphorylatable; and LglASA ($Lgl^{S656A, S660, S664A}$) which – from the consensus sequence – should be insensitive to Aurora. These varied forms also served as controls for our assay: that the consensus site around a particular serine is important, rather than that phosphorylation of these sites is unspecific in the presence of a kinase.

As expected, aPKC is able to phosphorylate LglSSS but not LglAAA (data not shown). CENP-A is phosphorylated by Aurora A and B on Ser7 and was used as a positive control: mutation of this site to alanine prevents phosphorylation (Kunitoku et al., 2003; Zeitlin et al., 2001). We observed that both Aurora A and B are able to directly phosphorylate LglSSS, but not LglAAA (Figure 3.8 A).

We further checked the specificity of the Aurora-mediated phosphorylation using various Lgl peptides. From the consensus sequence, Aurora should be able to phosphorylate Lgl only on S656 and S664, but not S660 (Figure 3.8 B). Consistent with this, the LglASA peptide was not phosphorylated upon addition of Aurora A, and LglSAS showed similar phosphorylation levels to the wild type Lgl (Figure 3.8 C). Thus, the middle serine of the tripartite motif, S660, is not an Aurora target, and hence would not have showed phosphorylation in the presence of Aurora in the Wirtz-Peitz study (Wirtz-Peitz et al., 2008). Other Lgl peptides back up this analysis: LglASS and LglAAS show similar levels of phosphorylation – i.e. the presence of the additional S660 does not lead to further phosphorylation, showing specificity in this assay rather than promiscuous kinase activity. Preliminary analysis suggests that Aurora B is also able to phosphorylate S656 and S664 only (Figure A.3).

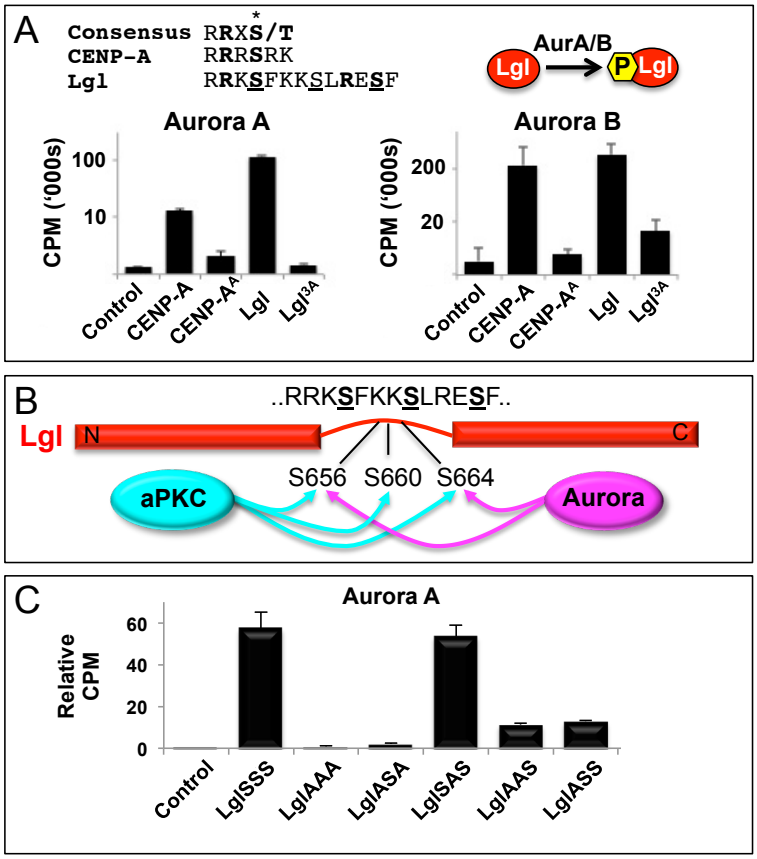

Figure 3.8 Aurora kinases directly phosphorylate Lgl *in vitro*

(A) Aurora A and B are able to directly phosphorylate an Lgl^{SSS} peptide, but not an Lgl^{AAA} (Lgl3A) peptide, in an *in vitro* kinase assay (mean ± SD, triplicate experiments). CENP-A was used as a positive control. Note that the Aurora consensus sequence matches the first and third serines in the Lgl tripartite motif.
(B) Schematic of aPKC and Aurora phosphorylation of the key Lgl tripartite motif. Aurora phosphorylates only S656 and S664.
(C) Elaboration of the *in vitro* kinase peptide assay with Aurora A (mean ± SD, triplicate experiments). Consistent with the consensus sequence and schematic in (B), LglASA is unable to be phosphorylated by Aurora A, and LglSAS shows similar phosphorylation levels to the wild type. LglAAS and LglASS show similar levels of phosphorylation, indicating further that S660 is not an Aurora target. Similar results were obtained with Aurora B (Figure A.3)

3.4.4 Attempted generation of a constitutively active Aurora construct

Similar to the suggested, but in our hands unsubstantiated, idea that expression of aPKC can drive Lgl into the cytoplasm (Grifoni et al., 2007) (Figure 3.6), we wondered whether the expression of Aurora would force the relocalisation of Lgl. Expression of Aurora A-GFP reveals its localisation as cytoplasmic and concentrated at centrosomes, but does not affect tissue architecture, cell division, or Lgl localisation (Figure A.4, (Berdnik and Knoblich, 2002)). Aurora A kinase activity is induced by phosphorylation of an activation loop (at threonine 295), and the binding of its partner Bora (Dodson and Bayliss, 2012; Hutterer et al., 2006). We attempted to generate a phosphomimetic and constitutively active Aurora A by mutating Thr295 to either Glutamic Acid (E) or Aspartic Acid (D). Neither of these constructs showed any gross morphology phenotype in wing or eye discs when expressed in clones or with ptc.GAL4, nor was Lgl localisation affected to any real noticeable extent (Figure 3.9 A, and data not shown). The co-expression of our Aurora A construct and its activator Bora also did not affect Lgl localisation (Figure 3.9 B-C). The activity of Aurora is thought to revolve also around feedback with Cdk1 activity (Van Horn et al., 2010), so it may be that our attempt to activate Aurora with a phosphomimetic construct was too crude, even with the presence of Bora. The substitution of Thr295 to D or E would also not guaranteed to cause constitutive activation, and we did not fully analyse the details of these constructs.

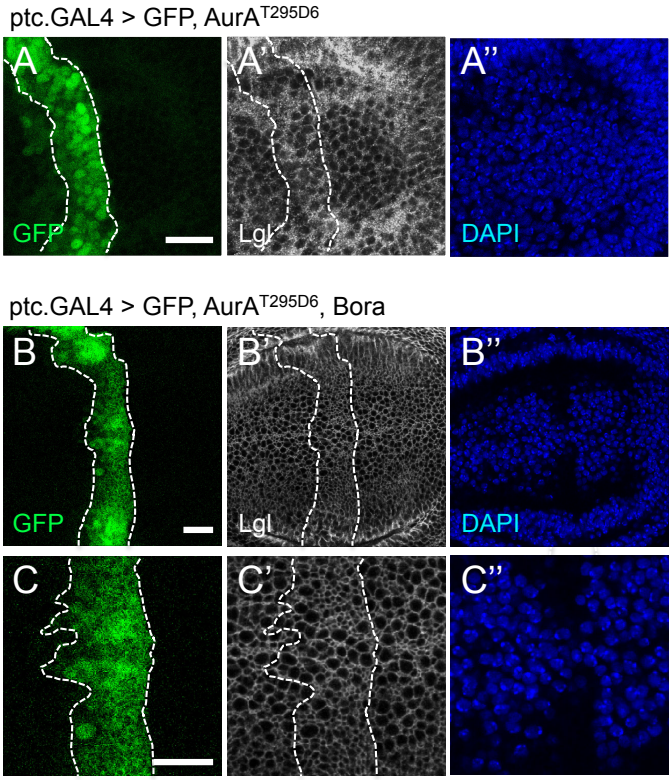

Figure 3.9 Expression of Aurora A^{T295D} does not force Lgl relocalisation

(A) Expression of Aurora A^{T295D} does not cause any change in Lgl localisation in wing discs. The additional presence of the Aurora A activator, Bora, also fails to induce Lgl relocalisation (B-C). Note that disc tissue architecture is unaltered, and thus the constructs may simply be inactive. Scale bars 20μm.

3.5 The Aurora kinases are semi-redundant in *Drosophila*

Although it is reported in the literature that both Aurora A and B can phosphorylate some targets (e.g. CENP-A, (Kunitoku et al., 2003; Zeitlin et al., 2001)), and this was validated in our kinase assay, very little work has been carried out on the extent of the redundancy between the two kinases. We therefore were interested to carry out some further analysis in this respect.

3.5.1 Redundancy of Aurora A and B using VX-680

As noted above, detection of phospho-Lgl is abolished in Western blotting in samples cultured with the dual Aurora kinase inhibitor VX-680. Phospho-Lgl stains mitotic cells in wild type, and this staining is still present in *aurora A* mutant or Aurora B RNAi cells: only in discs treated with VX-680 does phospho-Lgl staining not appear (Figure 3.10 A'-C', E'). In *aurora A* mutant discs, the centrosomal staining of phospho-Lgl is somewhat reduced, but cytoplasmic localisation remains. Aurora B RNAi cells grow to a very large size, as they are unable to complete cytokinesis. Both phospho-Lgl and Lgl-GFP are cytoplasmic in cells with multiple centrosomes, suggesting mitotic relocalisation still occurs (Figure 3.10 C', D).

We noticed when examining Lgl localisation in VX-680 treated discs that very few cells enter mitosis. Again we compared wild type, a*urora A* mutants, Aurora B RNAi expressing cells, and VX-680 treated discs (Movie M5). In the same time period, numerous cells undergo mitosis in a wild type disc. *aurora A* mutant cells do progress through mitosis, but at a dramatically slower speed (about 90 minutes from obvious cell rounding, compared to 20 minutes in wild type). Aurora B RNAi cells do somewhat round up (from their large size), but fail to undergo cytokinesis (see also Movie M17). In discs cultured in media containing VX-680, very few mitotic events are seen, although the tissue remains viable. Treating discs with VX-680 for several hours, and then transferring them to fresh media results in a temporary inhibition of most mitotic events, before the drug (presumably) wears off after a few hours and cell divisions occur again (data not shown).

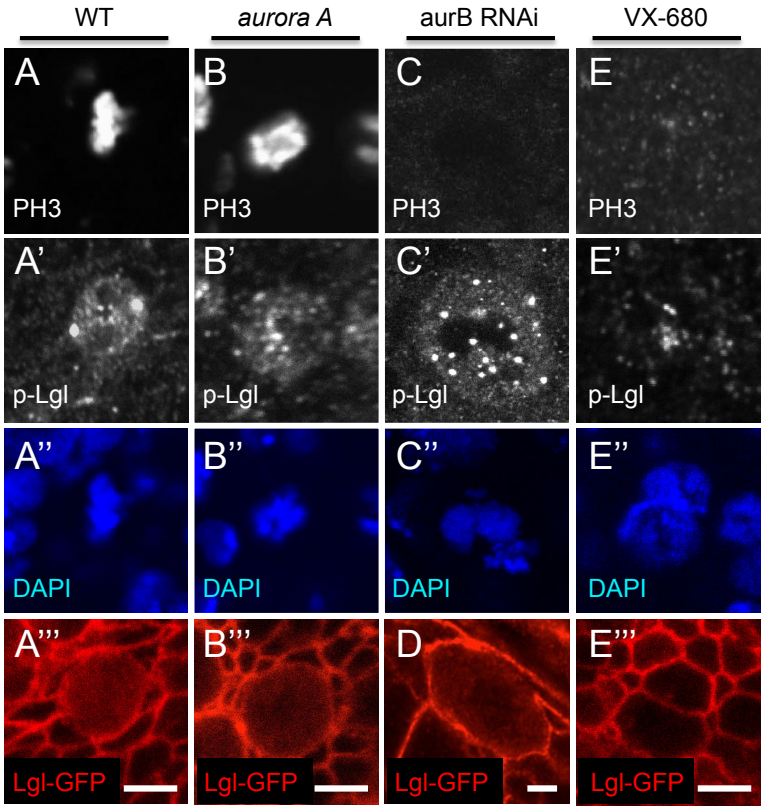

Figure 3.10 Treatment of epithelia with VX-680 reveals semi-redundancy between Aurora A and B kinases

(A) Phospho-Lgl antibody strongly accumulates in the cytoplasm of mitotic cells. Bright spots indicate centrosomes. Lgl-GFP relocalises to the cytoplasm. (B) Phospho-Lgl staining still accumulates and Lgl-GFP relocalisation still occurs in *aurora A* mutant discs. (C) Aurora-B RNAi mitotic cells are far larger in size relative to wild type, are polyploidy, and show increased centrosome numbers, due to defects in cytokinesis. Phospho-Lgl staining still accumulates and Lgl-GFP relocalisation still occurs. Note that PH3 staining is absent, as this is dependent on Aurora B kinase activity. (E) Phospho-Lgl staining is absent in VX-680 treated discs, although mitotic cells are still present, as indicated by cell size and interkinetic nuclear migration. Same image as Figure 3.7 D. Note that the centrosomal staining of the phospho-Lgl antibody is absent in mitotic cells in *aurora A* mutants, or VX-680 treated discs (B', E'). Scale bars 5μm.

3.5.2 Redundancy of Aurora A and B using genetic approaches

In using the dual kinase inhibitor drug, we noted that very few cells undergo or enter mitosis. We wondered therefore whether the two Auroras together could be required for mitotic entry in addition to their better-known functions; and that investigation into the effects of depletion of either form singly failed to pick up on this aspect due to the redundancy. We therefore attempted to investigate this by two genetic approaches.

First, we compared the size of mitotic clones (generated by the MARCM system) in wing discs and follicle cells. Compared to wild type clones, *aurora A* mutant clones tended to be slightly smaller (though detailed quantification was not carried out), and still contained multiple cells per clone (Figure 3.11B). Clones expressing Aurora B RNAi showed the established phenotype of very large cells due to cytokinesis defects, but still were recoverable and also showed multiple cells per clone (Figure 3.11 C). In contrast, clones of *aurora A* mutant cells also expressing Aurora B RNAi rarely contained more than one or two cells, consistent with the idea that these clones could not enter mitosis (Figure 3.11 D-E). Note that Aurora B RNAi clones presumably do essentially progress through mitosis in order for the increase in cell size and polyploidy to occur: but clonal cells in the double *aurora* depletion experiment remained normal size. The experiment showed some similar results in the follicle cell epithelium, but not all clones expressing Aurora B RNAi showed the large cell phenotype (Figure 3.11 F-I). It may be that the effect of the RNAi came on too late in this tissue, and thus the wing disc results appear more accurate.

We also examined the presence of cells expressing GFP under the control of the *ptc* promoter in wing discs. In wild type discs, a stripe is seen down the middle of the disc (Figure 3.11 J). A similar pattern is seen in *aurora A* mutant discs. We note that the disc architecture is again fairly normal in *aurora A* mutant discs, and there is an increased mitotic index as shown by PH3 staining (Figure 3.11 K). Cells expressing GFP and Aurora B RNAi under the *ptc* promoter are still present in the disc, and display the large cell phenotype (Figure 3.11 L). However, cells

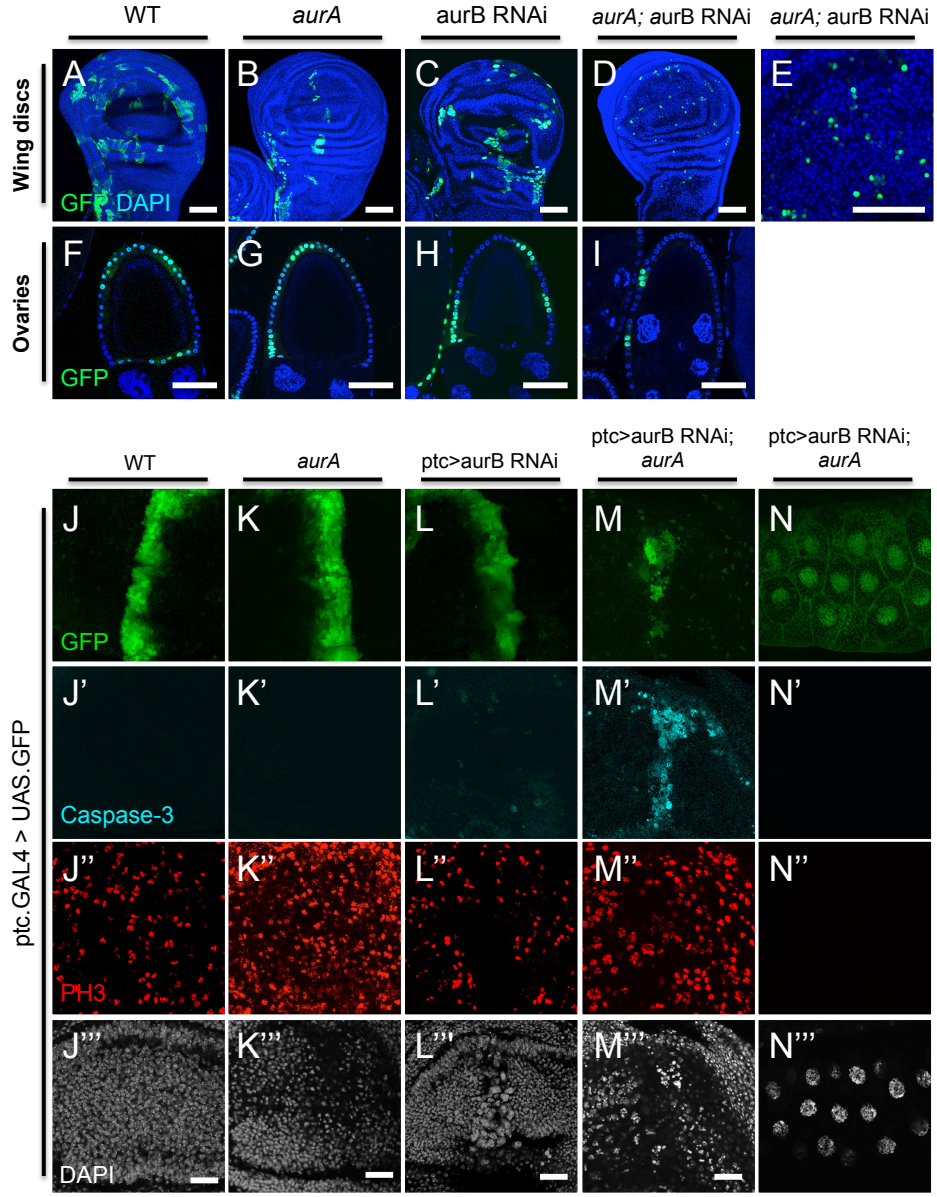

Figure 3.11 Aurora A and B are semi-redundant in *Drosophila* epithelia

(A-E) MARCM clones expressing GFP in wing discs. Compared to wild type (A), clones of *aurora A* are slightly smaller, but still easily recovered (B). Clones of Aurora B RNAi cells show large polyploidy nuclei, but still can be found in groups of multiple cells (C). In contrast, cells depleted of both Aurora A and B are mostly found in single cell clones. Zoom in (E).
(F-I) Similar results are found in follicle cell epithelium, though the Aurora B RNAi effect seems weaker, since not all cells expressing the RNAi show polyploidy nuclei.
(J-N) Wing discs expressing GFP under the control of ptc.GAL4. In wild type discs, the GFP stripe is fairly uniform, and no cell death is seen (J). *aurora A* mutant discs demonstrate elevated mitotic index as shown by PH3 staining, but no cell death or altered GFP patterning (K). Aurora B RNAi expression leads to large cells and nuclei, but still no cell death or perturbed GFP pattern (L). Cells expressing Aurora B RNAi in an *aurora A* mutant disc undergo apoptosis as shown by Caspase-3 staining, and the GFP pattern is perturbed as cells die (M).
(N) Aurora B RNAi expressing cells in *aurora A* mutant salivary glands do not show evidence of cell death or disrupted tissue structure. Since cells of the third instar larvae salivary gland do not undergo mitosis, this suggests a purely mitotic effect of the Aurora depletion. Scale bars 50μm (A-I), 20μm (J-M)

expressing Aurora B RNAi in an *aurora A* mutant disc begin to die, as shown by Caspase-3 staining, and the pattern of GFP expression is smaller and less uniform (Figure 3.11 M). This resultant cell death appears mitosis specific, because cells expressing Aurora B RNAi in an *aurora A* background in the salivary gland, which does not undergo mitosis, do not die or show elevated Caspase-3 expression (Figure 3.11 N). It is not entirely clear why double Aurora depletion cells should be eliminated in such a way, but this phenotype mimics that of mutations in *string*, a phosphatase required for mitosis (Neufeld et al., 1998). Presumably these cells are eliminated by cell competition due to failure to enter mitosis.

In our experiment of double *aurora* depletion in clones in the wing disc, we observed single cell clones, but no obvious cell death. This is likely to be due to the time point of the generation of these mitotic clones: had they been induced at an earlier stage (e.g. with a heat-shock 48-hours after egg-laying, in contrast to 72-hours), we would perhaps expect these cells to also be eliminated.

3.6 LglASA is polarised correctly but does not relocalise in mitosis

Our data so far suggested that Aurora, rather than aPKC, is responsible for the mitotic relocalisation of Lgl. The consensus sequence of Aurora and the kinase assays we performed implied that phosphorylation only occurred on two of the three serines in the tripartite motif (S656 and S664, but not S660). We have shown that Lgl3A – with all three serines mutated to non-phosphorylatable alanine – no longer relocalises during mitosis, and is not correctly polarised in epithelia. We reasoned that a construct of LglASA – that is, S656A, S660, S664A – should still be able to be phosphorylated by aPKC and thus be polarised; but not phosphorylated by Aurora, and so not relocalise to the cytoplasm during mitosis. If so, this construct would lend support to the distinction between Aurora- and aPKC-mediated regulation of Lgl, and perhaps provide a tool to dissect the mitotic role of Lgl from that of its functions in polarity.

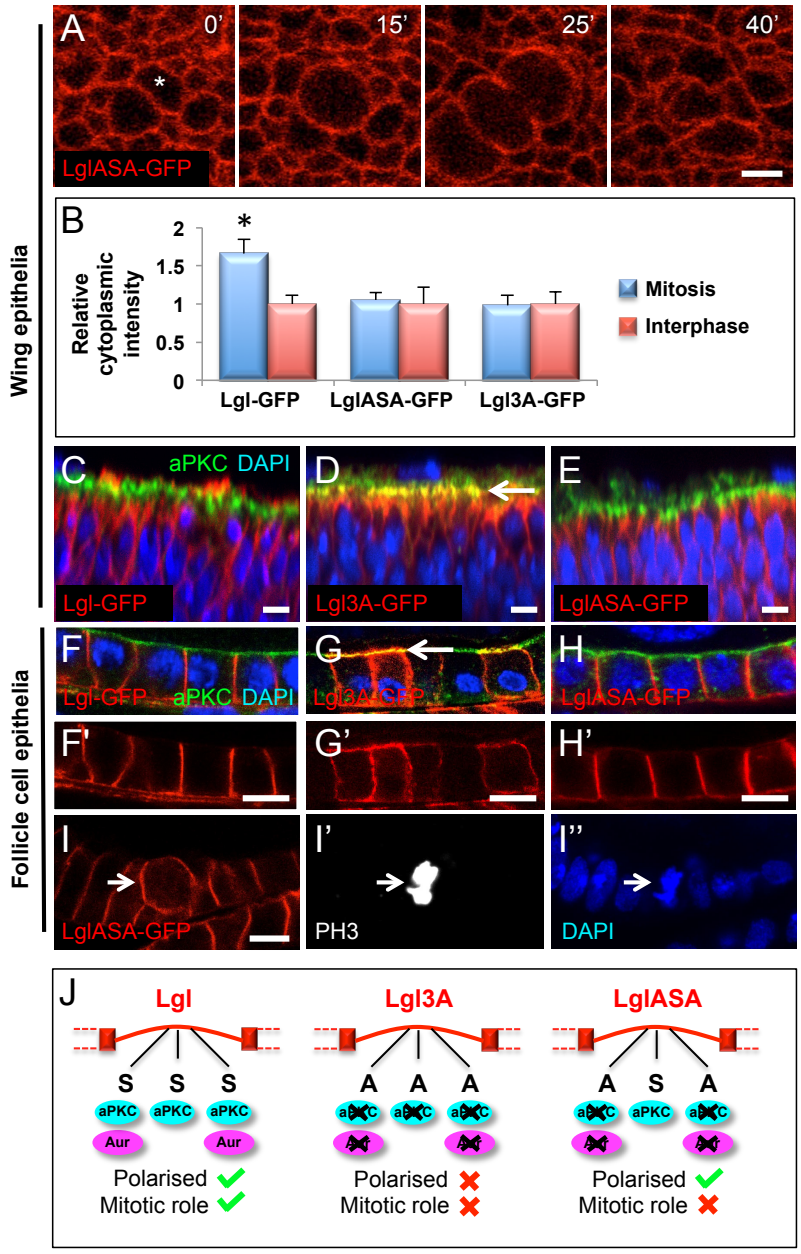

Figure 3.12 LglASA fails to relocalise to the cytoplasm in mitosis, but is polarised similar to wild type in interphase

(A, I) A construct of LglASA-GFP, which is insensitive to Aurora phosphorylation, remains cortical throughout mitosis, similar to Lgl3A. Quantification in B (mean ± SD, n≥10 for Lgl-GFP and Lgl3A-GFP, n≥50 for LglASA-GFP). LglASA also does not relocalise in mitotic follicle cells (I). Quantification in Figure A.1. Statistical analysis of mitotic vs. interphase cells was performed using a two-tailed t test. An asterisk denotes a p value < 0.05.
(C-H) Wild type Lgl-GFP is removed from the apical membrane by phosphorylation by aPKC, and thus does not co-localise with aPKC in wing discs or follicle cells (C, F). Non-phosphorylatable Lgl3A remains/spreads apically and co-localises with aPKC at the apical membrane of cells (arrows) (D, G). LglASA-GFP resembles wild type polarisation, and does not spread apically or co-localise with aPKC in epithelial cells (E, H).
(J) Schematic of regulation of Lgl by aPKC and Aurora kinases, and speculated functions of Lgl. Since LglASA is still polarised, but does not relocalise to the cytoplasm in mitosis, it should be useful to help dissect the role of Lgl's mitotic relocalisation. Scale bars 5μm. See Movie M6.

We therefore generated both GFP and non-GFP tagged LglASA constructs, and looked for their localisation in interphase and mitotic cells. Consistent with our expectations, LglASA did not relocalise during mitosis, instead remaining at the cortex, similar to Lgl3A-GFP (Figure 3.12 A-B, Figure A.5, and Movies M6 and M15). However, while Lgl3A co-localises with aPKC at the apical membrane in wing disc or follicle cell epithelia, LglASA is fully absent from the apical membrane, and does not overlap with aPKC (Figure 3.12 C-H). Although LglASA therefore appears insensitive to phosphorylation by Aurora, the remaining serine (S660) is presumably still phosphorylated by aPKC, and leads to removal of LglASA from the apical membrane and thus polarisation in interphase cells similar to wild type. Similar results of LglASA behaving like wild type Lgl were obtained in neuroblasts in third instar larval brains, which is discussed with in Chapter 4. We next sought to use the LglASA construct to investigate any roles for Lgl in mitosis, distinct from its functions in polarity.

3.7 Summary

We found that in wing disc epithelia, polarity is largely maintained as cells progress through mitosis. The basolateral polarity determinant Lgl, a neoplastic tumour suppressor in *Drosophila*, relocalises from the cortex to the cytoplasm in a phosphorylation-dependent manner. The regulation of this mitotic relocalisation is not dependent on aPKC, which is involved in removing Lgl from the apical membrane; but on the cell cycle kinases Aurora A and B. Aurora A and B show semi-redundancy *in vivo* and may be required for mitotic entry. Aurora A and B directly phosphorylate a peptide of Lgl on two conserved serine residues: S656 and S664. A transgenic construct with these two sites mutated to alanine no longer relocalises during mitosis, but is still correctly polarised, presumably because aPKC can phosphorylate the remaining serine of the tripartite motif, S660. The generation of this construct allowed us to try and dissect apart the functions and phenotypes of Lgl in cell polarity, and its role in mitosis (Figure 3.12 J). The next chapter details the investigation into the role of Lgl's mitotic relocalisation in both symmetric and asymmetric cell division.

Chapter 4. The roles of Lgl in symmetric and asymmetric division

4.1 Proposed functions of Lgl in mitosis

Much of the research on Lgl has focussed on its role in polarity and antagonism of aPKC. This is in part because of the dramatic phenotype of *lgl* mutant larvae, which fail to pupate, and continue to grow as larvae for several days beyond wild type, eventually dying as giant larvae. The imaginal discs lose cell polarity and tissue organisation, forming giant amorphous masses, and clones of *lgl* cells in follicle cells similarly show loss of polarity and form multi-layered masses (Bilder et al., 2000; Gateff, 1978). Recent work on *dlg* and *scribble*, the two other members of the basolateral Scribble module and also neoplastic tumour suppressors, has made use of specific techniques and mutants to investigate roles for these proteins that are separate to the polarity functions (Bergstralh et al., 2013b; Nakajima et al., 2013). We anticipated that the generation of the LglASA construct, in addition to a myristoylated Lgl construct that is tethered to the membrane, would allow us to perform similar investigations into the mitotic role of Lgl and the distinction from its role in cell polarity.

The main role suggested recently for Lgl in mitosis is as a molecular buffer for aPKC kinase activity in asymmetric cell division, helping to tightly control the regulation of cell fate determinants (Wirtz-Peitz et al., 2008). Another report suggested that *lgl* depletion in mammalian cell culture results in mitotic spindle disorganisation, through a direct binding to LGN, the mammalian homologue of Pins (Yasumi et al., 2005). We took a general approach to try to elucidate the role of Lgl in mitosis in epithelia, and used our constructs to probe more delicately the proposed functions.

4.2 Is the cytoplasm a temporary dumping ground for Lgl in mitosis, or a region for specific activity?

We had two general hypotheses for the reason behind Lgl relocalisation. The first is a desire to remove Lgl from the cell membrane for some reason, and therefore to temporarily displace Lgl into the cytoplasm throughout mitosis. This general principle holds true for some planar cell polarity determinants, which are asymmetrically localised in a cell prior to division. In order to ensure symmetric division, the asymmetric components are internalised in mitotic cells, and re-established asymmetrically after cell division (Devenport et al., 2011). The second hypothesis is that Lgl has some specific role in the cytoplasm of the cell, and must be relocalised in order to perform this function.

In neuroblasts, expression of Lgl3A - which is non-phosphorylatable and therefore remains on the membrane during mitosis – is reported as perturbing the correct asymmetric localisation of cell fate determinants like Miranda (Atwood and Prehoda, 2009; Lee et al., 2006a). This is likely due to affecting aPKC activity, which is directly responsible for phosphorylating Miranda (and others). Thus in this system, it seems important to remove Lgl from the membrane, rather than a specific role in the cytoplasm.

In our work so far, we had expressed Lgl3A-GFP in wing discs and follicle cell epithelia (whilst investigating the more proximate causes of Lgl relocalisation), and not seen any obvious dominant effects on cell division (Chapter 3): timing, spindle organisation or orientation and daughter cell size all appeared normal. Similarly, the expression of the LglASA-GFP construct, which remains cortical during mitosis, did not have any effect that we could see. We also used an Lgl-GFP construct with a myristoylation tag, retaining Lgl at the cortex (myrLgl-GFP). This construct remains cortical during mitosis in wing disc or follicle cell epithelia, and is localised around the whole cell cortex, including the apical domain (Figure 4.1). Similarly to LglASA and Lgl3A, expression of myrLgl did not show any dominant effects in wing discs or follicle cells (Figure 4.1). Whilst we would come back to this question in more detail later, we thus initially thought that the Lgl would be required in the cytoplasm for a particular reason, rather than simply needing to be removed from

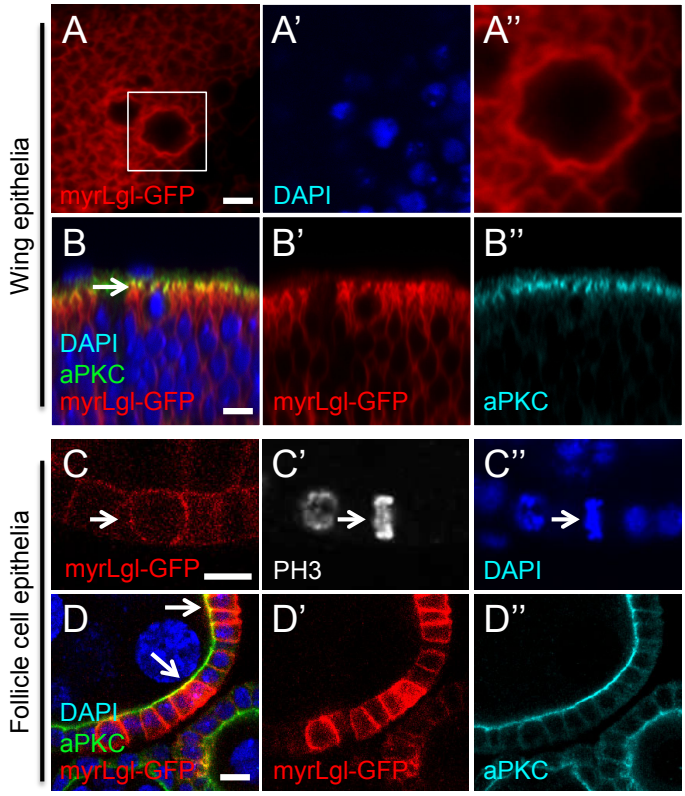

Figure 4.1 myrLgl remains cortical during mitosis

A construct of Lgl containing a myristoylation tag (myrLgl) remains cortical during mitosis and does not relocalise to the cytoplasm in wing disc epithelia or follicle cells (A, C), quantification in Figure A.1. myrLgl is membrane-tethered around the entire cell cortex, and colocalises with aPKC at the apical membrane in wing disc (B) or follicle cell (D) epithelia. Note that the expression of myrLgl does not perturb cell polarity. Scale bars 5μm (A-C), 10μm (D).

the cortex. The phospho-Lgl antibody used had also shown enrichment at centrosomes, and we tentatively considered this as a region for Lgl function.

4.3 *lgl* mutants show mitotic defects *in vivo*

A report in mammalian cell culture had suggested that Lgl2 (one of the two mammalian homologues of *Drosophila* Lgl) is involved in spindle assembly, probably through its direct binding to LGN (Pins) (Yasumi et al., 2005). The authors had noted that depletion of *lgl2* by RNAi resulted in spindle disorganisation and multi-nucleated cells. We wondered whether we would be able to see similar defects in *Drosophila*.

We found that *lgl* mutant discs (lgl^{4}/lgl^{334}) showed disorganised mitotic spindles compared to wild type (Figure 4.2 C, F-G). Since *lgl* discs overgrow and show severe disruptions to polarity and tissue structure, as a control we also examined *dlg* mutant discs, which show similar overgrowth and loss-of-polarity phenotypes. Interestingly, although spindles were sometimes misoriented compared to the plane of the epithelium in *dlg* (dlg^{M52}/dlg^{1}) discs, spindle organisation appeared similar to wild type (Figure 4.2 E, Figure 4.3 B). Although Dlg is established as having a role in spindle orientation (Bergstralh et al., 2013b), in this experiment we could not separate such a role from the general disorganisation of the disc. We also noted that *lgl* mutant discs show some monopolar spindles, in contrast to wild type or *dlg* mutant discs (Figure 4.3 A-C).

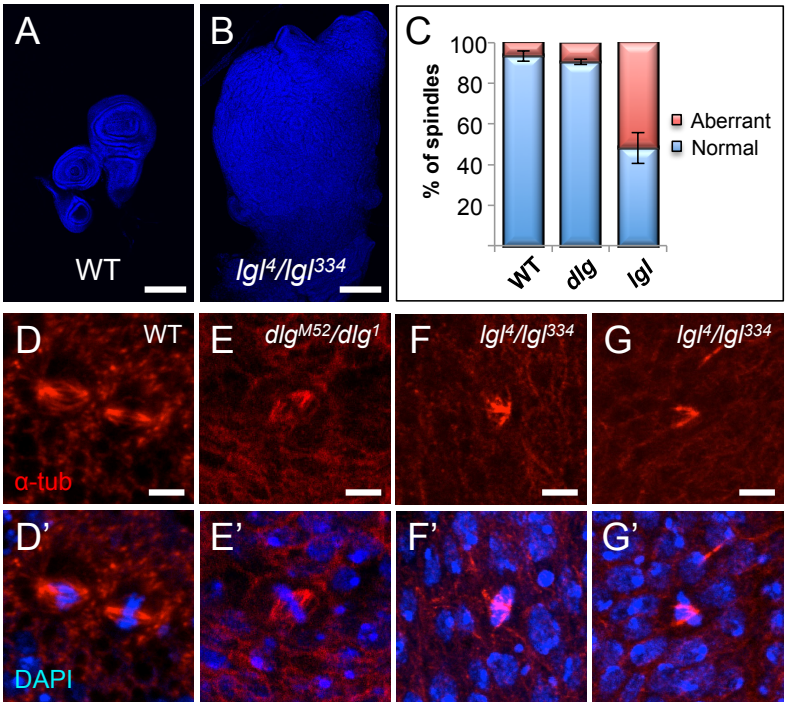

Figure 4.2 Mitotic spindle assembly defects in *lgl* mutants *in vivo*

(A-B) *lgl* mutant discs lose polarity and tissue organisation, eventually growing much larger than wild type.
(C-G) *dlg* mutant discs, which also lose polarity and overgrow similar to *lgl*, show spindle organisation similar to wild type (D-E). Spindles in *lgl* mutant discs are often disorganised and monopolar (F-G). Quantification in C (mean ± SD; n=65 (WT), 59 (*dlg*), 56 (*lgl*)). 'Aberrant' spindles were counted as spindles not resembling wild type: such as monopolar and disorganised. Scale bars 200μm (A-B), 5μm (D-G).

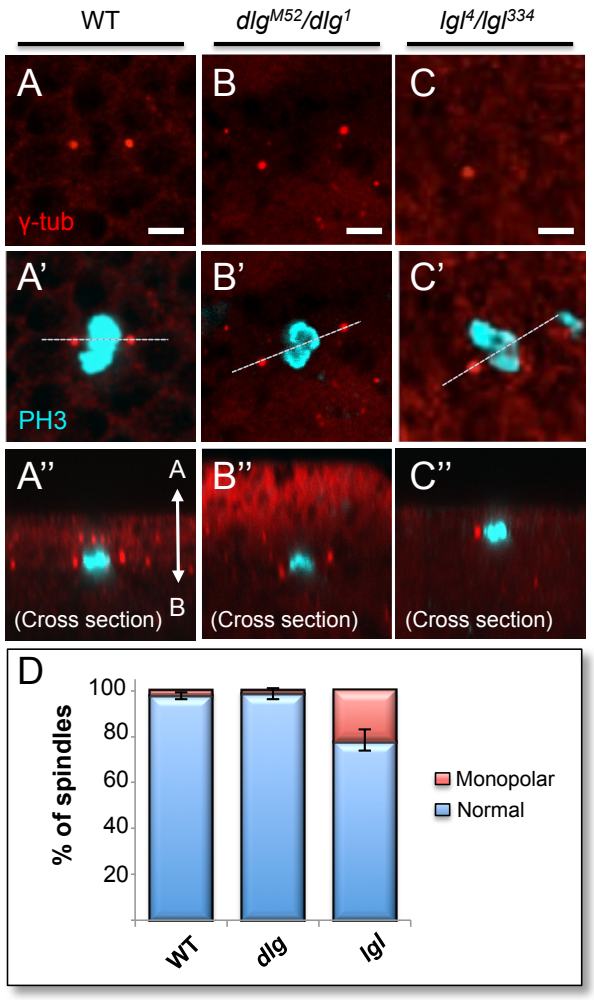

Figure 4.3 Mitotic defects in *lgl* mutants *in vivo*

Mitotic cells in wild type discs have two centrosomes, which lie parallel to the plane of the epithelium (A-A''). Mitotic cells in *dlg* mutant discs also show regular spindles as staining by γ-tubulin, although they are not always aligned with the plane of the epithelium, due to loss of tissue architecture, or spindle orientation defects (B-B''). In addition to spindles not lying parallel to the disc epithelium, mitotic cells in *lgl* mutant discs sometimes appear monopolar (C-C''). Quantification in D (mean ± SD, n=79 (WT), 77 (*dlg*), 193 (*lgl*)). Scale bars 5μm.

We attempted to confirm these observations by live imaging *lgl* mutant discs (Figure 4.4 and Movies M7-M9). We observed similar results to the fixed staining: some mitotic cells appeared normal, and some showed defects. We noted that in cases of mitotic defects, the metaphase plate and spindle organisation often appeared normal at some stages, before displaying aberrant effects. We observed that mitosis often proceeded slower than in wild type discs, and noted some seemingly monopolar/"splitting" spindles (Figure 4.4 B), spindles seemingly unable to align themselves correctly in the cell (data not shown), or, when mitosis proceeded at a speed comparable to wild type, lagging chromosomes (Figure 4.4 C). These results confirmed the data from fixed stainings – note the similarity between the spindles in Figure 4.2 F and Figure 4.4 B (24') - and seemed consistent with a role for Lgl in the cytoplasm affecting spindle assembly

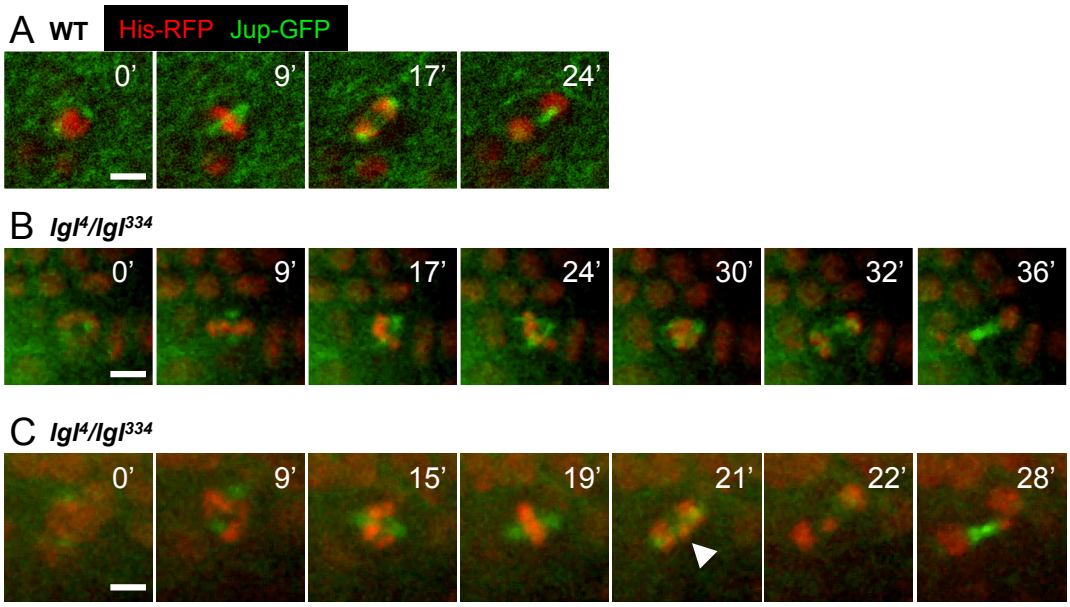

Figure 4.4 Lgl is required for efficient spindle function *in vivo*

(A) Still frames from mitosis in wild type wing discs expressing His-RFP and Jup-GFP to mark DNA and tubulin.
(B) Mitotic often proceeds at a slower speed in *lgl* mutant discs. Despite fairly efficient initial formation of the metaphase plate (9'), mitosis does not proceed similar to wild type cells, and the spindle 'splits' (24'). Anaphase does still occur, but much later than in wild type (32').
(C) When mitosis occurs at the same speed as wild type, other defects appear in *lgl* mutant discs. Despite the appearance of a normal metaphase plate (19') the previous time points betray a difficultly in spindle organisation. As anaphase proceeds, timely chromosome segregation fails (21').
Time 0' is taken as the first point at which it is possible to accurately identify which cell will proceed through mitosis. See Movies M7-M9. Cell cycle timing defects were not quantified due to low 'n' numbers, and timings here represent the individual movies. Scale bars 5μm.

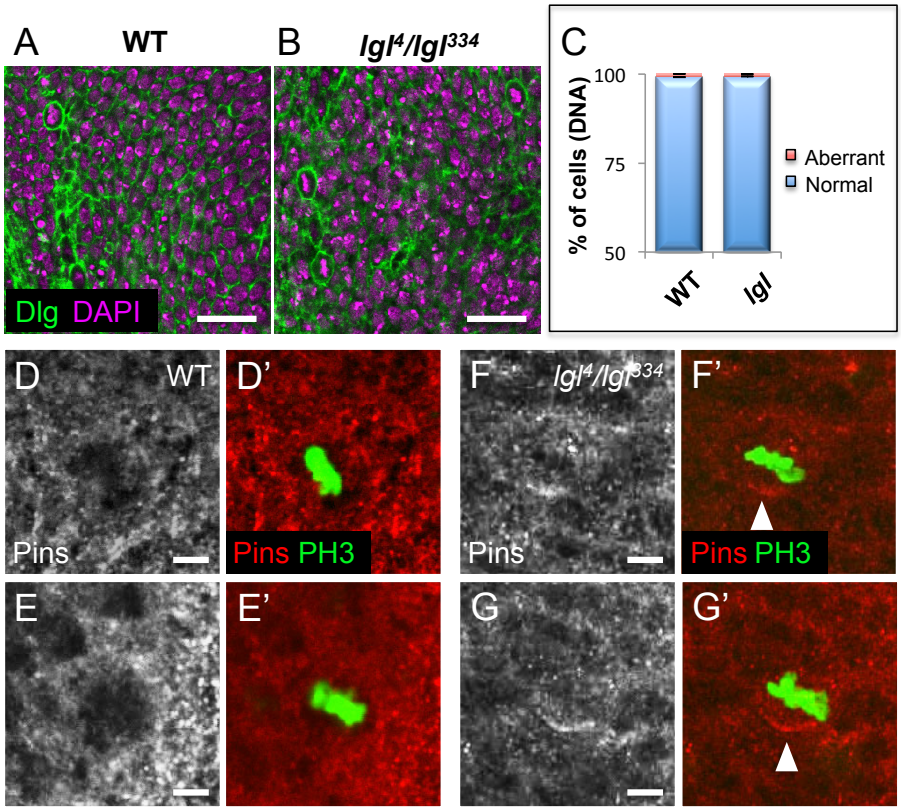

Figure 4.5 Analysis of *lgl* mutant wing discs

(A-C) Comparison of the nuclei of wild type and *lgl* mutant wing discs. There is no noticeable increase in multinucleate cells or otherwise aberrant nuclei in *lgl* mutants (mean ± SD, n=235 (WT), 484 (*lgl* mutant).
(D-G) Pins stainings in wild type wing discs is unlocalised (D-E). There may be slight cortical enrichment of pins around mitotic cells in *lgl* mutant discs (F-G, arrowheads). Scale bars 20μm (A-B), 5μm (D-G).

We performed a similar analysis of *lgl* depleted cells as Yasumi et al (Yasumi et al., 2005), by looking for multinucleated cells in whole mutant wing discs; but did not find any differences compared to wild type (Figure 4.5 A-C). The effect observed by Yasumi et al was very mild (Yasumi et al., 2005), wing disc cells are small, and there is some cell death occurring in *lgl* mutant discs, so the absence of any noticeable effect is not particularly remarkable.

Lgl2 was reported to bind directly to LGN in mammalian cell culture, and proposed to strengthen the interaction between LGN and NuMA (Pins and Mud in *Drosophila*) (Yasumi et al., 2005). We therefore wondered whether we could see any effect on Pins in *lgl* mutant discs. LGN is reported as being phosphorylated by aPKC in cell culture (Guilgur et al., 2012; Hao et al., 2010), and Pins is supposedly absent from the apical membrane in *aPKC temperature sensitive* mutant alleles in the *Drosophila* wing disc (Guilgur et al., 2012). Pins is also reported as being phosphorylated by Aurora A for a function in mitosis (Johnston et al., 2009), somewhat similar to our findings with Lgl. We did not obtain clear results for any effect on Pins in *lgl* mutant discs, though there may be some slight cortical enrichment Figure 4.5 (D-G). The lack of polarisation in the *lgl* mutant discs, combined with poor stainings with the antibody we used precludes us from making any detailed comments on this.

4.4 Non-relocalising Lgl constructs rescue cell polarity, but not cell proliferation, in wing disc epithelia

Previous studies on members of the Scribble module have used particular techniques or mutations in order to separate effects of polarity from mitotic roles (Bergstralh et al., 2013b; Nakajima et al., 2013). In addition to the LglASA construct generated for this work, a myrLgl construct had previously been generated in the lab. This myrLgl construct is membrane-tethered and does not relocalise to the cytoplasm during mitosis; additionally, although myrLgl spreads apically and colocalises with aPKC, it does not disrupt cell polarity (Figure 4.1). We therefore sought to employ the LglASA and myrLgl constructs, to provide further insight into a mitotic role for Lgl, distinct from a role in polarity. We performed rescue

experiments, where LglASA or myrLgl would be expressed in *lgl* mutant clones, in order to isolate effects of non-relocalising Lgl.

First, in wing discs, we confirmed that myrLgl and LglASA remain cortical during mitosis: note that the effect is more striking on the edge of a clone (Figure 4.6 A-C). We noted that the clone size of these rescues was significantly smaller than *lgl* clones rescued by (wild type) Lgl-GFP in wings discs (Figure 4.6 D-G) or ovaries (Figure 4.6 H-K). Since LglASA is localised basally, similar to wild type Lgl, we did not expect any polarity defects in a rescue with this construct. Similarly, although myrLgl-GFP spreads apically, it does not perturb cell polarity when expressed (Figure 4.1). LglASA is localised normally (Figure 3.2; further experiments were performed on this theme, see Figure 4.8), thus we did not suppose the clone size to be a product of cell polarity defects, which leads to clonal elimination. We found similar results in follicle cell epithelia, which may suggest against the idea that small clones result due to apoptotic cell death.

To test this further, we performed another rescue experiment; this time expressing the Lgl construct in the entire posterior compartment of *lgl* mutant wing discs using the hedgehog.GAL4 driver. Expression of Lgl-GFP rescued cell polarity in the posterior compartment as expected, and roughly half the disc – i.e. the posterior compartment – is rescued (Figure 4.7 A). Expression of myrLgl or LglASA in the posterior compartment did rescue cell polarity, but this compartment was reduced in size compared to that in the wild type rescue (Figure 4.7 B-E).

We noted in the wing disc, *lgl* mutant clones rescued with LglASA or myrLgl showed spindles misoriented relative to the plane of the epithelium. Similar results were found in the posterior compartment of *lgl* mutant discs expressing LglASA or myrLgl under the control of the hedgehog promoter. (Figure 4.7 F-H, Figure A.6). Misorientation was also observed in *pins* or *mud* mutant discs, two proteins that have established roles in spindle orientation. *mud* mutants showed more severe phenotypes than either *lgl* mutant rescue experiments or *pins,* suggesting some distinct roles for Mud in spindle orientation, discussed in Chapter 6. Spindle misorientation has previously been described for the basolateral polarity determinants Dlg and Scribble (Bergstralh et al., 2013b; Nakajima et al., 2013).

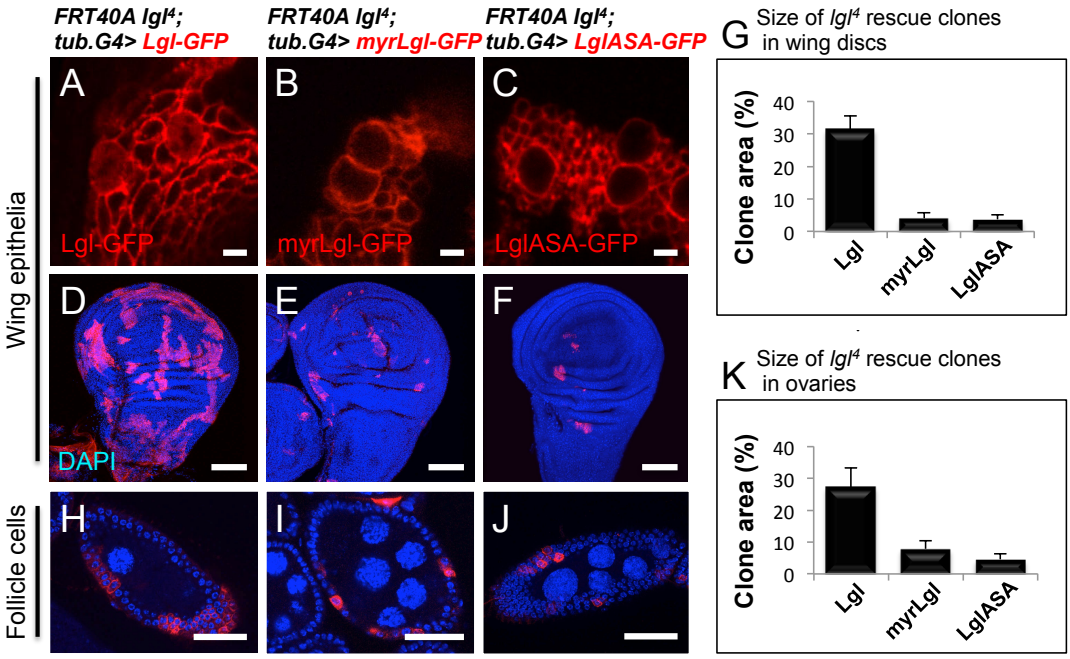

Figure 4.6 Non-relocalising Lgl constructs rescue cell polarity but not clone size

(A-G) Whilst clones of *lgl*^{4} expressing Lgl-GFP in the wing disc generate large clones (A, D; n=9 wing discs), *lgl*^{4} mutant clones expressing myrLgl-GFP (B, E; n=9 wing discs) or LglASA-GFP (C, F; n=10 wing discs) generate small clones. Quantification in G; mean values ± SD. Note that myrLgl-GFP (B) and LglASA-GFP (C) do not relocalise efficiently to the cytoplasm at mitosis. Similar results were obtained in the follicle cell epithelium (H-K, mean ± SD; n=15 ovarioles (Lgl-GFP), 17 (myrLgl), 14 (LglASA). Scale bars 5μm (A-C) 50μm (D-F, H-J).

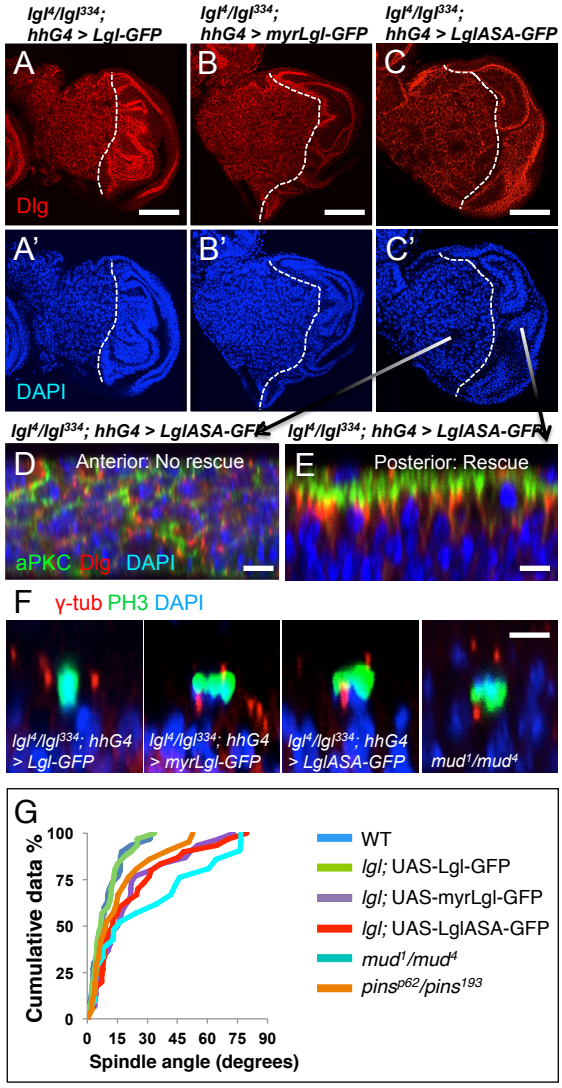

Figure 4.7 Constructs of non-localising Lgl rescue cell polarity, but reveal spindle orientation defects in wing disc epithelia

(A-C) *lgl*^{4}/*lgl*^{334} mutant wing discs expressing Lgl-GFP, myrLgl-GFP or LglASA in the posterior compartment under the control of hhGal4. Expression of Lgl-GFP in the posterior compartment results in a rescue of cell polarity defects, and approximately half the disc being rescued (A). Although polarity defects are rescued upon expression of myrLgl-GFP or LglASA in *lgl* mutant discs, the rescue (posterior) compartment is smaller than the Lgl-GFP rescue.
(D-E) Cross-section through anterior (D) and posterior (E) regions of *lgl*^{4}/*lgl*^{334} mutant discs expressing LglASA in the posterior compartment. The anterior compartment (no rescue) shows the typical loss of polarity and tissue architecture seen in *lgl* mutant discs (D). Note that polarity is completely rescued upon the expression of LglASA in the posterior compartment (E).
(F-G) *lgl* mutant discs that express myrLgl-GFP or LglASA-GFP show misoriented mitotic spindles relative to the plane of the epithelium, in contrast to those rescued with Lgl-GFP. *mud* mutant discs also show spindle misorientation phenotypes. Quantification in G. Spindle angles in metaphase cells were measured relative to the plane of the epithelium and plotted in rank order from lowest to higher. Spindle angles in *lgl* mutant cells rescued with LglASA (n=30, $p<0.05$) or myrLgl (n = 30, $p<0.05$), or spindles in *mud* mutant discs (n=21, $p\leq0.01$) differ significantly from those in the wild type or *lgl* mutants rescued with Lgl-GFP (n=32, ns compared to wild-type). p values as determined by the Kolmogorov-Smirnov test. Scale bars 50μm (A-C), 5μm (D-F).

If Aurora phosphorylates Lgl, and the prevention of this phosphorylation (e.g. LglASA) can lead to spindle orientation defects, then *aurora* mutant cells might show similar effects. However, the classic *aurora* mutant phenotypes of single centrosomes in mitotic cells, 'rosettes' of DNA in mitotic cells, and multiple free centrosomes precludes any useful analysis in this respect (Figure A.7). Note that spindle orientations were only quantified in cells with clear bipolar and organised spindles. Spindle misorientation effects can appear milder in wing discs than follicle cells, and we therefore performed similar Lgl rescue experiments in this tissue (Figure 4.8).

4.5 Aurora-insensitive Lgl rescues cell polarity in the follicle cell epithelium but reveals mitotic spindle orientation defects

Clones of cells mutant for the basolateral polarity determinants *lgl*, *dlg* or *scribble* form multi-layered masses of cells with disrupted polarity. Similar to the wing disc rescue experiments, expressing Lgl-GFP, myrLgl-GFP or LglASA-GFP in *lgl* mutant clones in the follicle cell epithelium restored cell polarity and clones did not become multi-layered (Figure 4.8 A-D).

Mitotic cells in follicle cell epithelia typically divide parallel to the orientation of the epithelial layer (Figure 4.8 E). *lgl* mutant clones or cells rescued by wild type Lgl-GFP similarly divide parallel to the interphase cells (Figure 4.8 F-H). In contrast, *lgl* clones or cells rescued by LglASA or myrLgl – which therefore express only cortical Lgl and have no Lgl in the cytoplasm – show misoriented mitotic spindles (Figure 4.8 I-L), similar to those published for *dlg* and *pins* (Bergstralh et al., 2013b). We also checked whether simply expressing LglASA-GFP or myrLgl-GFP would have an effect on spindle orientation in a wild type background, and could find no effect (quantification shown in Figure 4.8 M).

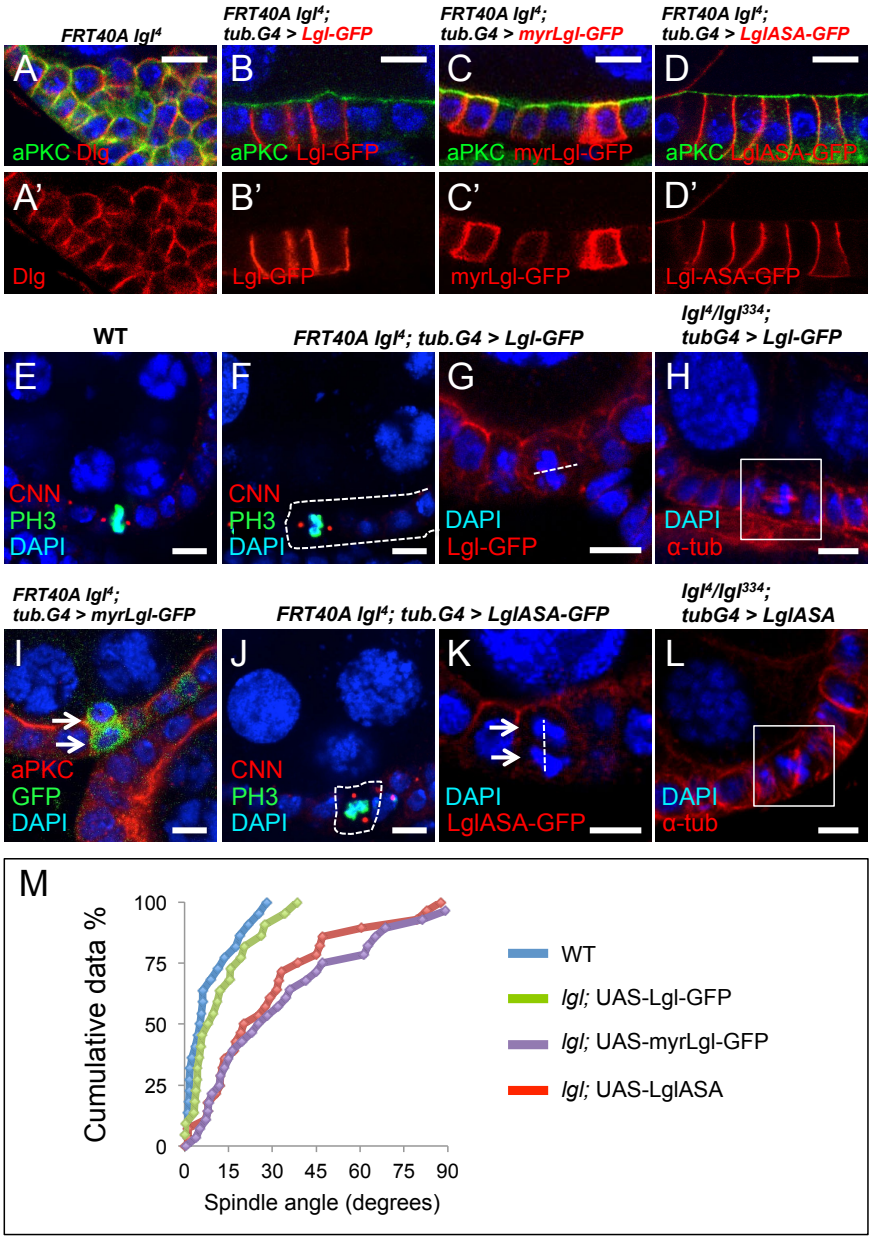

Figure 4.8 Aurora-insensitive Lgl rescues cell polarity but reveals defects in mitotic spindle orientation in follicle cell epithelia

(A-D) Clones of *lgl*^{4} mutant cells show multilayering and loss of polarity (A). This can be rescued by the expression of Lgl-GFP (B), myrLgl-GFP (C) or LglASA-GFP (D).
(E-M) Mitotic spindles in wild type discs are oriented roughly parallel with the plane of the epithelium (E). Clones of *lgl* mutant cells expressing Lgl-GFP show spindles oriented correctly (F-H), but expression of myrLgl-GFP (I) or LglASA-GFP (J-L) in *lgl* mutants results in misoriented spindles. Expression of LglASA-GFP in a wild type background does not cause spindles to become misoriented. Quantification shown in M. Spindle angles in metaphase cells were measured relative to the plane of the epithelium and plotted in rank order from lowest to higher. Spindle angles in *lgl* mutant cells rescued with LglASA (n=29, $p<0.01$) or myrLgl (n=28, $p<0.01$) differ significantly from those in the wild type or *lgl* mutants rescued with Lgl-GFP (n=22, ns compared to wild-type). p values as determined by the Kolmogorov-Smirnov test. Scale bars 10μm

4.6 Aurora-insensitive Lgl constructs rescue cell polarity and asymmetric division defects in neuroblasts

The initial literature suggesting aPKC phosphorylates Lgl to relocalise it to the cytoplasm was based on work in neuroblasts (Betschinger et al., 2005; Betschinger et al., 2003; Wirtz-Peitz et al., 2008): we therefore decided to re-examine the regulation and role of Lgl in this system. In mitotic neuroblasts, aPKC forms an apical crescent and restricts cell fate determinants such as Miranda and Numb to the basal side of the cell via phosphorylation events ((Wirtz-Peitz et al., 2008), Figure 4.9 A-B) We first expressed our various forms of Lgl to look at their localisation and any effect of over-expression. We find that Lgl-GFP behaves as previously described, being removed from the entire plasma membrane during mitosis, and does not affect aPKC or Miranda (Figure 4.9 C, (Betschinger et al., 2003; Wirtz-Peitz et al., 2008)). Lgl3A-GFP also behaves as described, localising around the membrane but not affecting aPKC localisation (data not shown). The Aurora-insensitive LglASA-GFP behaves differently to these, being removed apically but remaining localised at the basal membrane during mitosis (Figure 4.9 D, F-G). We found also that myrLgl remains cortical during mitosis, and aPKC colocalises with it around the entire plasma membrane (Figure 4.9 E). These results are consistent with the idea that aPKC is required for polarisation of Lgl away from the apical plasma membrane (and thus restricting it basally), while Aurora kinases are responsible for the relocalisation of Lgl from the plasma membrane into the cytoplasm. We note that in previous work, Lgl-GFP is transiently polarised basally, before it becomes completely cytoplasmic (Wirtz-Peitz et al., 2008), consistent with a dual regulation of Lgl. The removal of Lgl from co-localising and being in a complex with aPKC then allows aPKC to phosphorylate cell fate determinants required for asymmetric cell division. As such, expression of LglASA did not perturb aPKC or Miranda localisation (Figure 4.9 D), whereas expression of myrLgl caused aPKC and Miranda to spread around the cell (Figure 4.9 E).

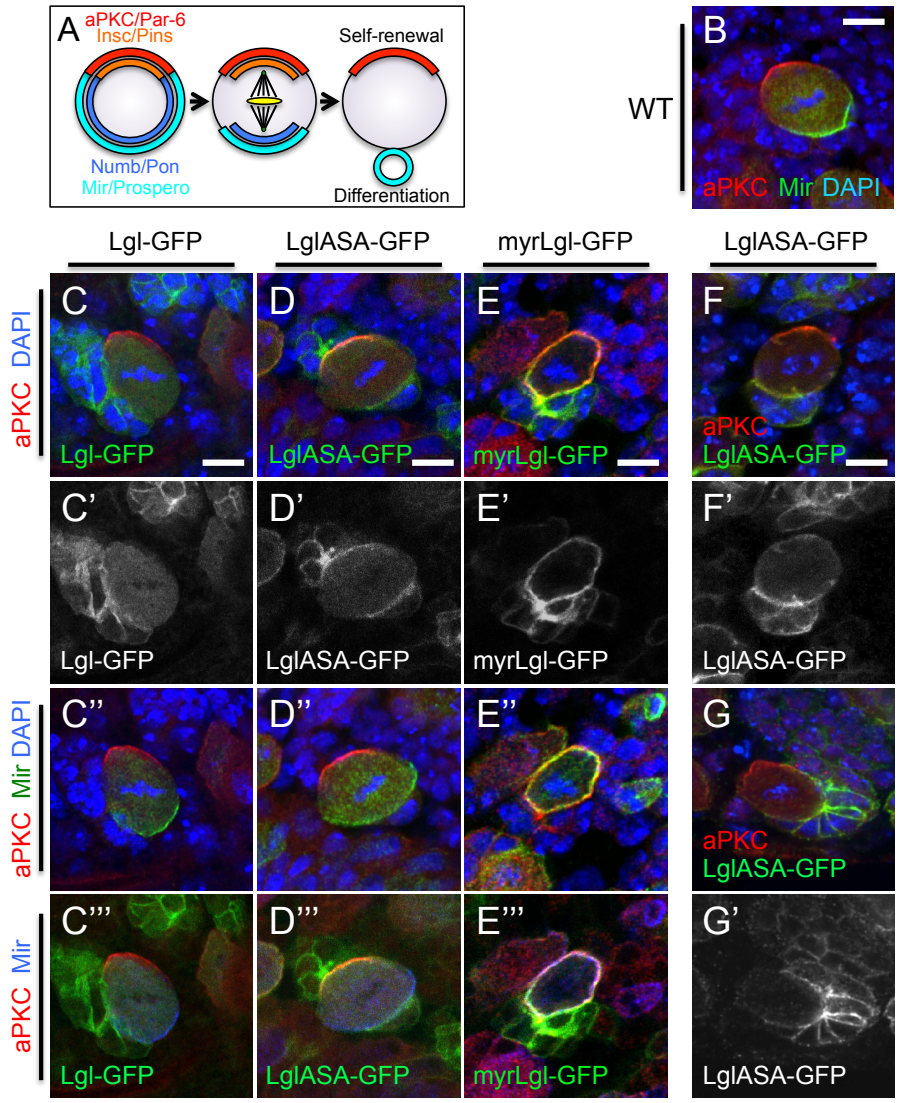

Figure 4.9 Localisations and effects of various Lgl constructs in neuroblasts

(A) Schematic of asymmetric cell division in neuroblasts. In wild type cells, aPKC localises apically and Miranda is phosphorylated and restricted to the basal domain (B).
(C-F) Lgl-GFP is cytoplasmic in metaphase neuroblasts, and aPKC and Miranda are correctly localised to the apical and basal sides respectively (C''). LglASA-GFP is polarised to the basal side of the neuroblast (D', F', G') and does not affect aPKC or Miranda localisation (D'', F, G). MyrLgl-GFP is localised around the entire cell cortex (E'), and its expression causes both aPKC and Miranda to spread around the cortex (E''). Scale bars 5μm.

We next examined the extent to which the various Lgl constructs could rescue *lgl* mutants in the larval brain. As previously published, we find that *lgl* clones display ectopic aPKC localisation, which leads to relocalisation of Miranda to the cell cortex (Figure 4.10 A). The expression of either Lgl-GFP or LglASA-GFP in lgl^4 mutant clones was sufficient to rescue Miranda polarisation and also spindle orientation (Figure 4.10 B-C). Thus, the polarisation of Lgl away from the apical membrane (as in LglASA), but not the total relocalisation to the cytoplasm, is sufficient for asymmetric cell division. The activity of aPKC is sufficient to restrict Lgl to the basal membrane, and therefore allows the unhindered activity of aPKC apically: resulting in correct polarisation of Miranda and other substrates basally, and normal asymmetric cell division. Consistent with this, whilst lgl^4 mutant clones display multiple neuroblasts within a single clone, indicating perturbed asymmetric division (because aPKC/Mir/Numb localisation is affected) (Figure 4.10 E), *lgl* clones expressing Lgl-GFP or LglASA (marked by CD8-GFP) display only one neuroblast per clone (Figure 4.10 F-G).

The expression of myrLgl in *lgl* mutant clones – which spreads around the entire cell membrane - results in inhibited aPKC activity, as revealed by the spreading of Miranda (Figure 4.10 D). Similarly to *lgl* mutants, where altered Miranda expression leads to perturbed asymmetric cell division, *lgl* clones expressing myrLgl-GFP also show multiple neuroblasts per clone (Figure 4.10 H). In wild-type cells, the mitotic spindle orients along the apical-basal axis, as measured with respect to the apical domain marked by aPKC (Figure 4.11 A). The cytoplasmic localisation of Lgl in neuroblasts does not appear to have any role in spindle orientation, since *lgl* mutant clones rescued with LglASA-GFP or myrLgl-GFP showed similar spindle orientation compared to wild type (Figure 4.11 B-D). aPKC is mislocalised in the rescue experiment with myrLgl-GFP, but Baz is localised similar to wild type, and was therefore used to mark the apical domain (Figure 4.11 D).

Collectively, these results indicate that in neuroblasts, the key step is the polarisation of Lgl to the basal membrane, preventing it from acting as a buffer to aPKC; and that the Aurora-mediated relocalisation to the cytoplasm is dispensable for asymmetric division and spindle orientation in neuroblasts.

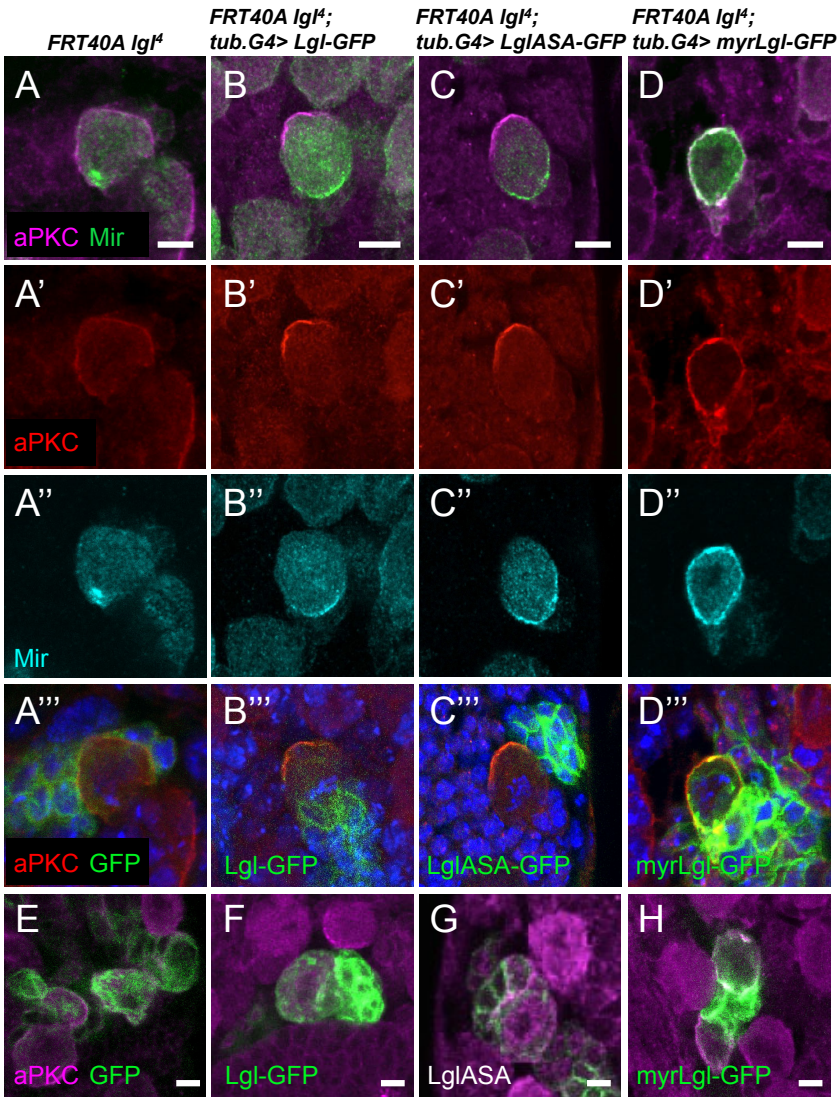

Figure 4.10 Aurora-insensitive Lgl rescues cell polarity and asymmetric cell division defects in larval brain neuroblasts

(A-D) aPKC and Miranda form apical and basal crescents respectively in wild type metaphase neuroblasts (see Figure 4.9). *lgl* mutant neuroblasts show spreading of aPKC and cytoplasmic Miranda (A). *lgl* mutant neuroblasts expressing Lgl-GFP (B) or LglASA-GFP (C) show normal aPKC and Miranda localisation. *lgl* neuroblasts expressing myrLgl show aPKC spreading around the cell cortex and localising with Miranda (D). Note that *lgl* neuroblasts show defects in asymmetric cell division, with more than one neuroblast per clone (E). *lgl* mutant neuroblasts expressing Lgl-GFP (F) or LglASA (G, marked by CD8-GFP) show normal asymmetric division as measured by neuroblast number. *lgl* neuroblasts expressing myrLgl also show perturbed asymmetric cell division, with multiple neuroblasts per clone. Scale bars 5μm.

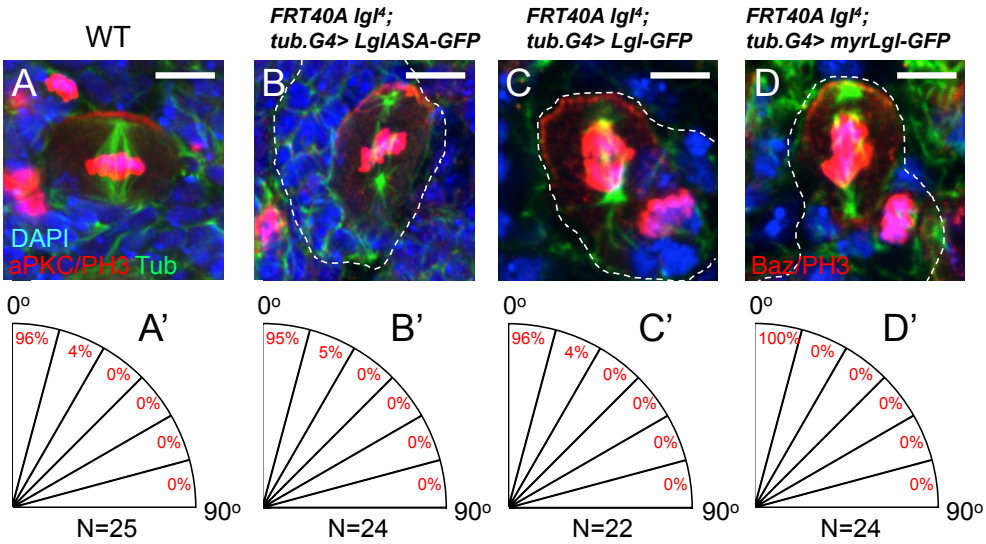

Figure 4.11 Cytoplasmic relocalisation of Lgl is not required for spindle orientation in neuroblasts

(A-D) Metaphase spindles in wild-type neuroblasts are oriented along the apical-basal axis, relative to the apical crescent of aPKC. Spindles in *lgl*^{4} mutant cells expressing Lgl-GFP (B), LglASA-GFP (C), or myrLgl-GFP (D) do not show any misorientation phenotypes. Metaphase cells are shown in (A-B), and anaphase cells in (C-D); quantifications were performed on metaphase cells only. Note that in (D), Baz was used to define the apical region, as aPKC is mislocalised in *lgl*^{4} cells expressing myrLgl-GFP. Scale bars 5μm.

4.7 Summary

We tried to investigate whether the relocalisation of Lgl was important because it removed Lgl from the membrane, or deposited it in the cytoplasm. Expression of membrane-bound Lgl showed no phenotype, and previous literature hinted at a cytoplasmic role. To dissect the polarity and mitotic functions of Lgl, we used the LglASA and myristoylated-Lgl constructs we generated. The LglASA construct, which can be phosphorylated by aPKC but not Aurora, remains cortical during mitosis but is polarised correctly, consistent with the notion that it is Aurora that phosphorylates Lgl to cause the cytoplasmic relocalisation in mitosis. Since LglASA is polarised normally, and does not affect aPKC localisation, it was not surprising to find that rescuing clones of *lgl* mutant cells with LglASA rescued polarity. Interestingly, orientation of the mitotic spindle was affected in these rescue experiments, and also when expressing myrLgl in lgl^4 mutant clones. Conversely, in neuroblasts, the polarity function of Lgl is more important, and as long as Lgl can be polarised to the basal domain – and so not affect aPKC activity – there is no detrimental effect on cell division. Thus the regulation of Lgl – being polarised by aPKC or fully relocalised to the cytoplasm by Aurora – has different importance in different tissues. The reasons and implications of this are discussed in Chapter 6.

Chapter 5. Cell polarity, mitosis and cancer

Many of the polarity determinants in epithelia are well described as being involved in tumour formation or progression (Ellenbroek et al., 2012; Macara and McCaffrey, 2013; Wodarz and Nathke, 2007). Historically, the members of the *Drosophila* basolateral Scribble module have perhaps had the most attention and are termed neoplastic tumour suppressors for their loss-of-function effects (Bilder, 2004; Bilder et al., 2000). Recent reports have described novel roles separate from polarity functions for Scribble and Dlg in spindle orientation, which may be linked to tumour progression (Bergstralh et al., 2013b; Nakajima et al., 2013). Our studies built on current questions within the field, and highlighted the lack of clarification in some areas.

5.1 Polarity and mitosis

5.1.1 Adherens junctions are down regulated during mitosis

Our studies had shown that Lgl is completely relocalised from the cortex to the cytoplasm during mitosis. Additionally, in follicle cell epithelia, aPKC is absent from the apical membrane during mitosis (Bergstralh et al., 2013b). We wondered whether the changes to polarity determinants in mitosis might hint at a susceptibility of mitotic cells to loss of polarity and any contribution to underlying biology of cancer progression.

One of the phenomena we noticed during mitosis was a down regulation of the adherens junctions: both E-cadherin and Armadillo/β-catenin are down regulated in mitotic cells relative in interphase cells in the wing disc epithelium (Figure 5.1 A-D and Movie M8). Bazooka/Par-3 shows similar, but milder, effects, consistent with its apical/junctional localisation. The adherens junctions down regulation is even more noticeable when two adjacent cells go through mitosis (Figure 5.1 B). We also performed transmission electron microscopy on wing discs to characterise this observation. In interphase, adherens junctions are easily recognisable in electron microscopy sections. In mitosis, consistent with confocal microscopy, adherens junctions appear less prominent (Figure 5.1 E).

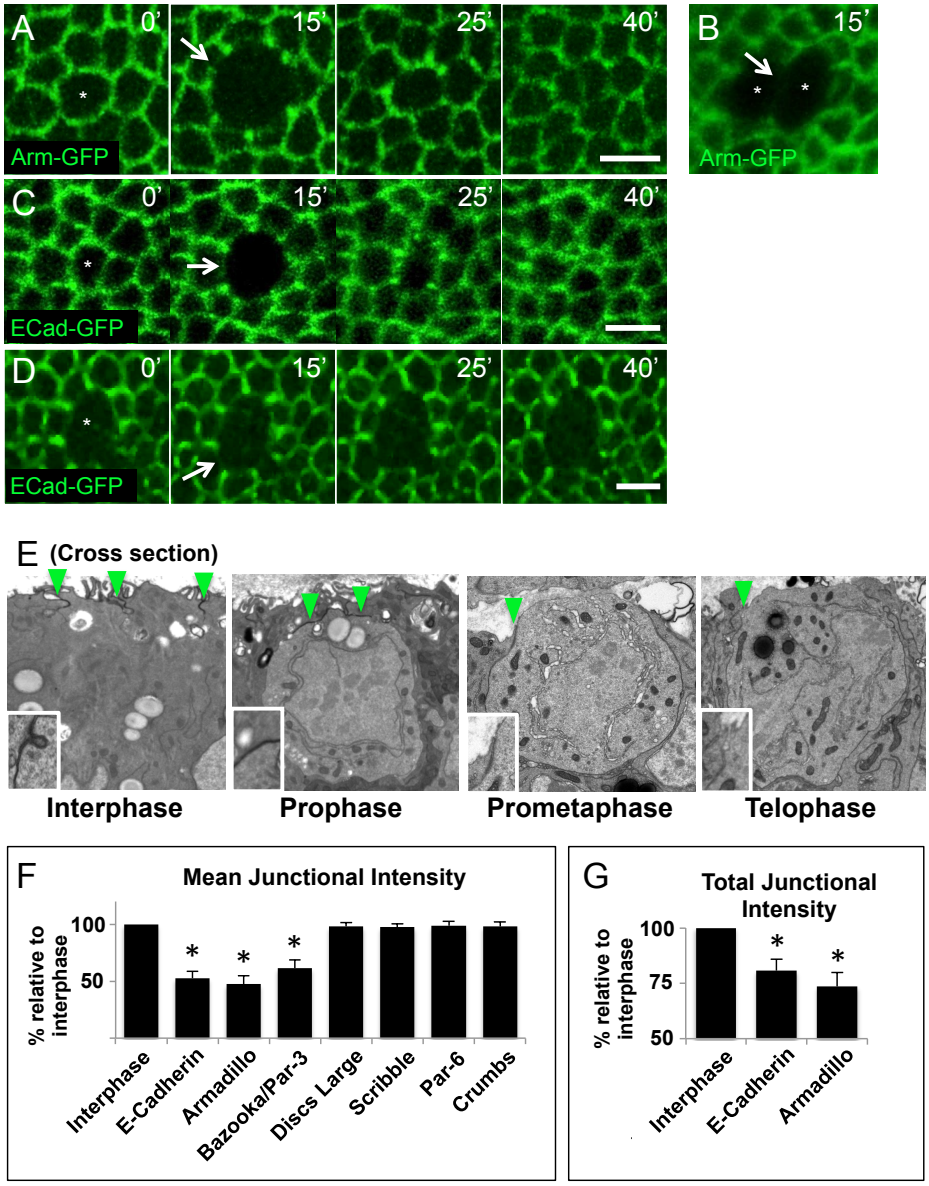

Figure 5.1 Adherens junctions are down regulated during mitosis

(A-D) Armadillo/β-catenin (A) and E-Cadherin (C) levels are reduced in mitotic cells (arrows). The down regulation is more noticeable in two adjacent mitotic cells (B). Arresting cells in metaphase with the microtubule inhibitor colchicine allows easy visualisation of the reduction in E-Cadherin (D).
(E) Transmission electron microscopy of wing discs. At interphase, adherens junctions are prominent (left picture, and inset box). As the cell progresses through mitosis and nuclear envelope breakdown, junctions become less striking ('Prometaphase' and inset). At telophase, presence of junctions is harder to see (right picture and inset).
(F) Quantification of mean polarity determinant intensity of cell-cell interfaces. Intensity of E-Cadherin, Armadillo and to a lesser extent Bazooka are lower at cell-cell interfaces in mitosis than in interphase cells. Intensity of other determinants (Dlg, Scribble, Par-6, Crb) is unaltered.
(G) Quantification of total junctional intensity around mitotic cells. Total levels of E-Cadherin and Armadillo as measured by fluorescence intensity are lower than interphase cells, rather than the signal simply being 'spread out' as the cell rounds up. Statistical analysis of mitotic vs. interphase cells was performed using a two-tailed t test. An asterisk denotes a p value < 0.05. See Movie M8. Scale bars 5μm.

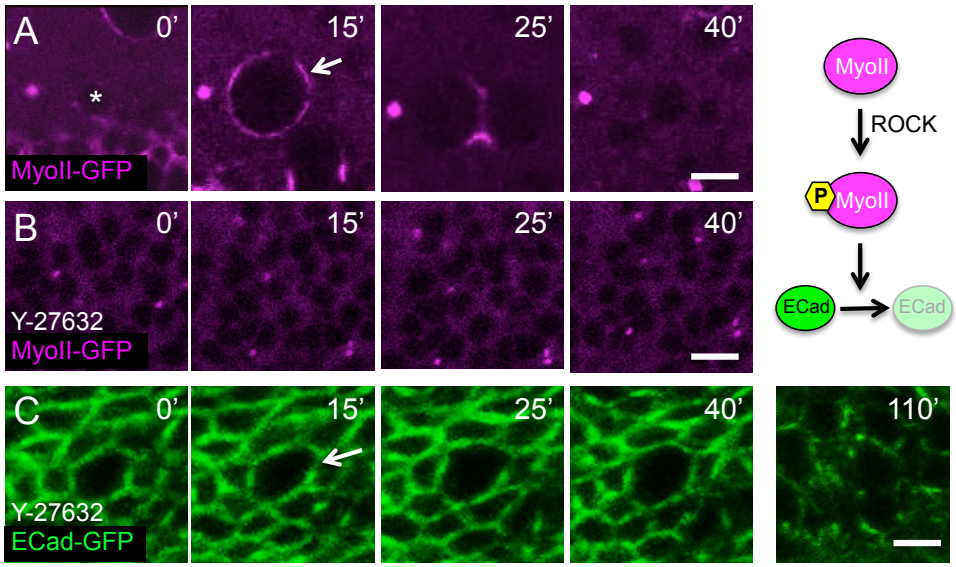

Figure 5.2 Rho-kinase controls adherens junction down regulation in mitosis

(A-B) MyoII-GFP accumulates around the cortex of mitotic cells and is also enriched at the cleavage furrow (A). Treatment of wing discs with the ROCK inhibitor Y-27632 prevents MyoII accumulation (B). Wing discs treated with Y-27632 no longer show down regulation of adherens junctions (C, arrow). After long-term incubation with the drug, tissue architecture is disrupted (C, 110'). See Movies M9 and M10. Scale bars 5μm.

Presumably junctions are not completely down regulated, and hence some junctions are still evident.

5.1.2 Rho-kinase controls adherens junction down regulation in mitosis

We next asked why and how junctions would be partially down regulated. Previously it had been described that the accumulation of Myosin could lead to the clustering and internalisation of E-Cadherin (Levayer et al., 2011). The cell rounding phenomena in mitosis occurs partly due to an accumulation of Myosin around the cell cortex, balancing the hydrostatic pressure from within the cell (Levayer et al., 2011; Stewart et al., 2011). Myosin-II accumulation and phosphorylation is attributed mostly via the action of Rho-kinase (Amano et al., 1996; Kimura et al., 1996; Levayer et al., 2011). We treated wing discs with the Rho-kinase inhibitor Y-27632, and failed to see any accumulation of Myo-II (Figure 5.2 A-B and Movie M11). Interestingly, we noted that E-Cadherin levels were no longer down regulated in mitosis upon Y-27632 treatment, despite cells still appearing to round up (Figure 5.2 C and Movie M12). After incubation with the drug for longer term, the tissue architecture was disrupted, consistent with large-scale abrogation of Myosin II (Figure 5.2 C, 110').

5.1.3 Aurora kinases can phosphorylate Myo-II but are not required for cell rounding

Live imaging of Lgl-GFP in *aurora A* mutant discs revealed that cell rounding was somewhat impaired, and the membrane appeared more fluid than in wild type, though cells were eventually rounded similar to wild type. Treating wing discs with the dual Aurora kinase inhibitor VX-680 resulted in a reduction in phospho-Myosin levels compared to wild type (Figure 5.3 A-D), but we were not sure whether this was a direct effect of Aurora on Myosin-II or an artefact of fewer cell divisions. Aurora B has been reported as being able to phosphorylate MRLC at Ser19 (Murata-Hori et al., 2000; Yokoyama et al., 2005), and we found that both Aurora A and B can phosphorylate a Sqh (MRLC) peptide *in vitro*, but not a Thr18/Ser19-Alanine phospho-mutant (Figure 5.3 F). Our results suggested a far stronger effect with Aurora B, consistent with the importance of Myosin in cytokinesis. We found

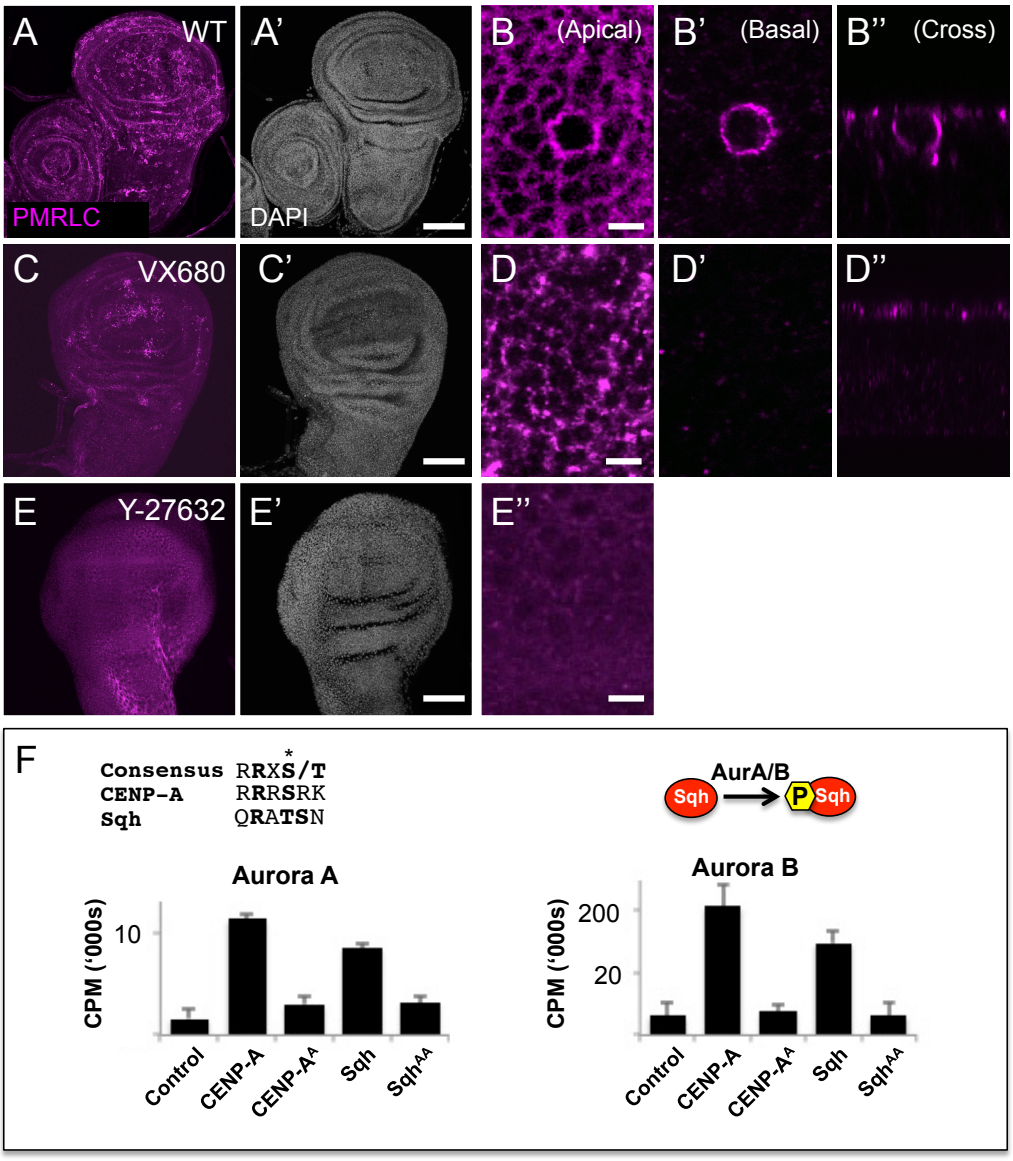

Figure 5.3 Aurora kinases can phosphorylate MLC but are not the key kinases responsible for p-Myo-II accumulation

(A-B) Phosphorylated Myo-II is present apically, and enriched around mitotic cells in wing disc epithelia. (C-D) Treatment with the dual Aurora kinase inhibitor VX-680 results in few mitotic cells, and thus absence of any basal p-Myo-II signal. Treatment of wing discs with the ROCK inhibitor Y-27632 results in no detectable p-Myo-II signal (E).
(F) Aurora A and B kinases can phosphorylate Sqh peptides (Myosin Regulatory Light Chain) *in vitro* (mean ± SD, triplicate experiments). Substitution of Thr18/Ser19 with alanine prevents phosphorylation. Note that phosphorylation by Aurora B is much stronger than by Aurora A. Scale bars 50μm (A, C, E), 5μm (B, D, E'').

that treatment of wing discs with the Rho-kinase inhibitor Y-27632 results in complete absence of p-Myo-II staining (Figure 5.3 E, (Meyer et al., 2011)). Thus although both Aurora A and B kinases can phosphorylate Myo-II *in vitro*, ROCK alone is sufficient for the Myo-II phosphorylation required in mitosis. The effects of the Aurora inhibition experiment on cell rounding may be down to an indirect effect on preventing ROCK activity, rather than direct phosphorylation. We note that that Aurora B is a critical regulator of cytokinesis, and its major role is probably phosphorylating Myo-II and Myo-II assistors in this process (Kondo et al., 2013; Wu et al., 2014). We speculate therefore that ROCK is the kinase primarily responsible for cell rounding, and Aurora B is additionally required for Myo-II phosphorylation for cytokinesis.

5.1.4 Requirement of adherens junction down regulation in mitosis

We next wondered why adherens junctions would be affected in mitosis. One hypothesis would be that the contact with neighbouring cells, mediated by junctions, would need to be somewhat relaxed as cells round up, in order to accommodate the local forces and dynamics within the tissue. If so, the prevention of down regulation of the adherens junctions components might be expected to cause defects in mitosis or the tissue. Treating wing discs with the Rho-kinase inhibitor did indeed result in fewer cells going through mitosis, and we tried to take a genetic approach to this question.

We tried expressing E-Cadherin, and looking at mitotic cells to see whether adherens junctions components were still affected, and if there was any effect on mitosis. Different alleles of E-Cadherin gave quite different effects when expressed - for example distorting the tissue morphology - and it was difficult to separate the global tissue effects from any specific mitotic effect (Figure A.8) Although it seems that expression of E-Cadherin prevented adherens junction down regulation, and we noted fewer mitotic cells, it is not clear whether this was a direct effect. We note that there have not been such strong reductions in E-Cadherin noted in other tissues (Founounou et al., 2013; Guillot and Lecuit, 2013; Herszterg et al., 2013). The distinction between interphase and mitotic cell morphology is wing discs is quite extreme, and tissue-specific effects may play a part here.

5.2 Sensitivity in mitosis of polarity determinant mutants

Our studies showed that both Lgl and adherens junctions components are altered during mitosis. We wondered whether mitotic cells would therefore be in a 'sensitised' state and be less resilient to any further perturbations to polarity or tissue stresses. We used the *Drosophila* eye disc as a model. In the eye disc, a wave of proliferation sweeps from posterior to anterior; cells posterior to the morphogenetic furrow are quiescent; cells anterior still proliferate.

In wild type eye discs, both proliferating and quiescent regions show normal cell polarity (Figure 5.4 A). In *lgl* mutant eye discs, we found that cell polarity was disrupted in the proliferating portion of the disc (anterior to the morphogenetic furrow, Figure 5.4 B''), but not where cells were quiescent (posterior, Figure 5.4 B'). This suggested that proliferating cells were somehow less able to cope with mutations in *lgl* than quiescent cells, and by some mechanism tend to lose cell polarity (or fail to maintain it).

In another experiment to test this, we noticed that mutant clones for many of the polarity determinants were mostly found in the posterior compartment of the eye disc: that is, where the cells were quiescent (Figure 5.5). Presumably, clones induced in the still-proliferating (anterior) part of the eye disc were eliminated, whereas those in the quiescent portion were not. Note that neither *crumbs* nor *bazooka* mutants resulted in elimination of clones, possibly due to redundancy between these two systems (Figure 5.5 F-G, (Thompson et al., 2013)). However, the recovery of mutant clones in the posterior, quiescent portion of the eye disc may be due to the perdurance of the proteins, rather than any other effect.

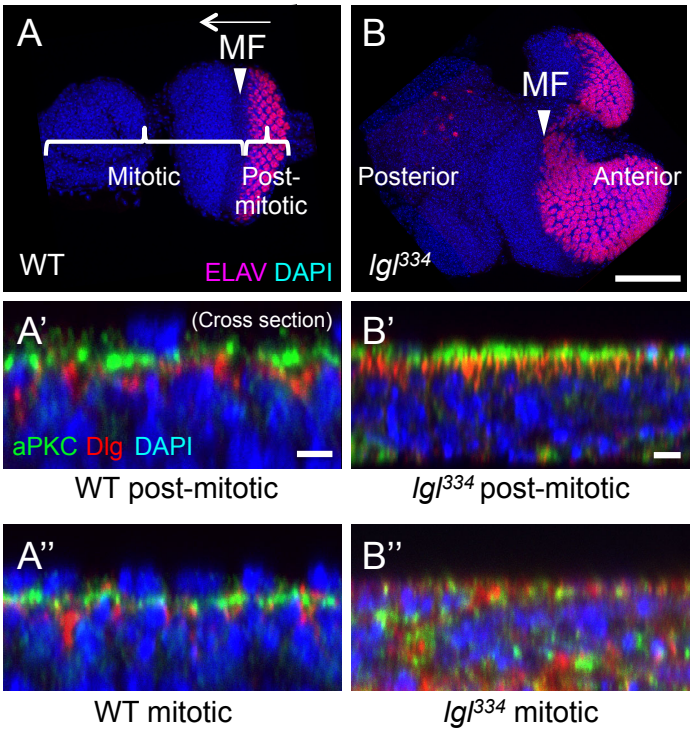

Figure 5.4 Mitotic epithelia are more sensitive to mutations in polarity determinants than quiescent epithelia

(A-B) Wild type and *lgl* homozygous eye discs. Note the large overgrowth in the *lgl* mutant (B). In wild type discs, all regions show normal cell polarity, regardless of cells being posterior to the morphogenetic furrow and quiescent (A'); or anterior and still proliferating (A''). Quiescent cells in an *lgl* mutant disc show normal cell polarity (B'), but proliferating cells in *lgl* mutant discs lose cell polarity (B''). Scale bars 100μm (A, B), 5μm (A', A'', B', B'').

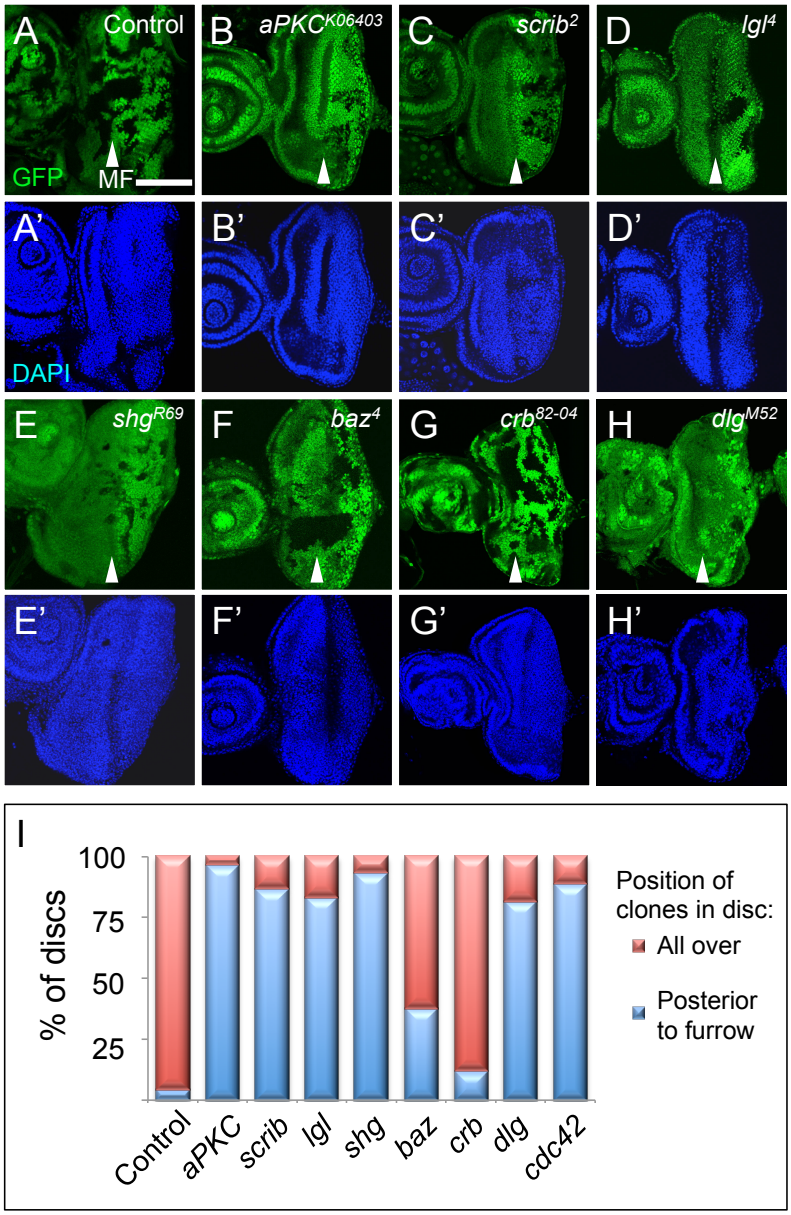

Figure 5.5 Proliferating epithelia are more sensitive to mutations in polarity determinants than non-proliferating epithelia

Clones of wild type cells in eye discs are found all over the disc (A), irrespective of the state of cell proliferation/quiescence (indicated by the relation to the morphogenetic furrow). Clones of cells mutant for polarity determinants are found nearly exclusively posterior to the morphogenetic furrow, where proliferation has ceased (B-E, H). Crumbs and Baz/Par-3 mutant clones are less restricted to the posterior, presumably due to redundancy (F-G). Quantification in I. n≥15 discs for each condition. Scale bars 100μm.

5.3 Driving proliferation causes cell elimination and polarity disruption

If cells are 'sensitive' during mitosis, we wondered whether forcing multiple cells into mitosis at the same time would have any effects on tissue polarity or organisation. Previously, it was reported that mitotic cells that internalise some polarity components then rely on their neighbouring cells to re-polarise correctly (Devenport et al., 2011). Since Lgl relocalises to the cortex after cell division in the daughter cells, we asked whether neighbouring cells could also be important here

We first forced over-proliferation in wing discs by expressing the E2F transcription factor and its partner Dp under the control of a ptc.GAL4 promoter (Figure 5.6). E2F transcription factors play key roles in the G1/S transition in the cell cycle, and transcriptional targets include cyclins, CDKs and cell-cycle checkpoint regulators. Cells expressing E2F were eliminated from the epithelium, dropping out basally and dying. (Figure 5.6 B) By co-expressing the cell death-preventer p35, cells still drop out, but are do not die: instead, they show aberrant polarity and morphology (Figure 5.6 C). Clones of forcibly proliferating cells show similar polarity perturbation, and the extent of elimination from the tissue depends on clone size (Figure 5.6 D). These results are reminiscent of experiments where cells eliminated from the epithelium (by spindle misorientation defects) usually die, but when kept alive with expression of p35, form tumour-like masses (Nakajima et al., 2013). Nakajima et al propose that this may be a contributing factor to tumour progression and invasive potential. Although the effect was striking, in our case it was not clear exactly what was causing cells to be eliminated from the epithelium. We therefore attempted to live image wing discs expressing E2F under ptc.GAL4 expression whilst also expressing E-Cadherin-GFP across the whole disc. We observed a dramatic increase in the number of mitotic cells in this region but it was not clear that mitotic cells were the ones being eliminated (Movie M16). Instead, we noted that cells in the E2F region tended to be much smaller, presumably due to the increased numbers of cells squeezed into a similar space. Apical constriction is a feature for cell delamination and removal from the epithelium (Marinari et al., 2012), and this is a likely explanation for the cause of the cell elimination in our case.

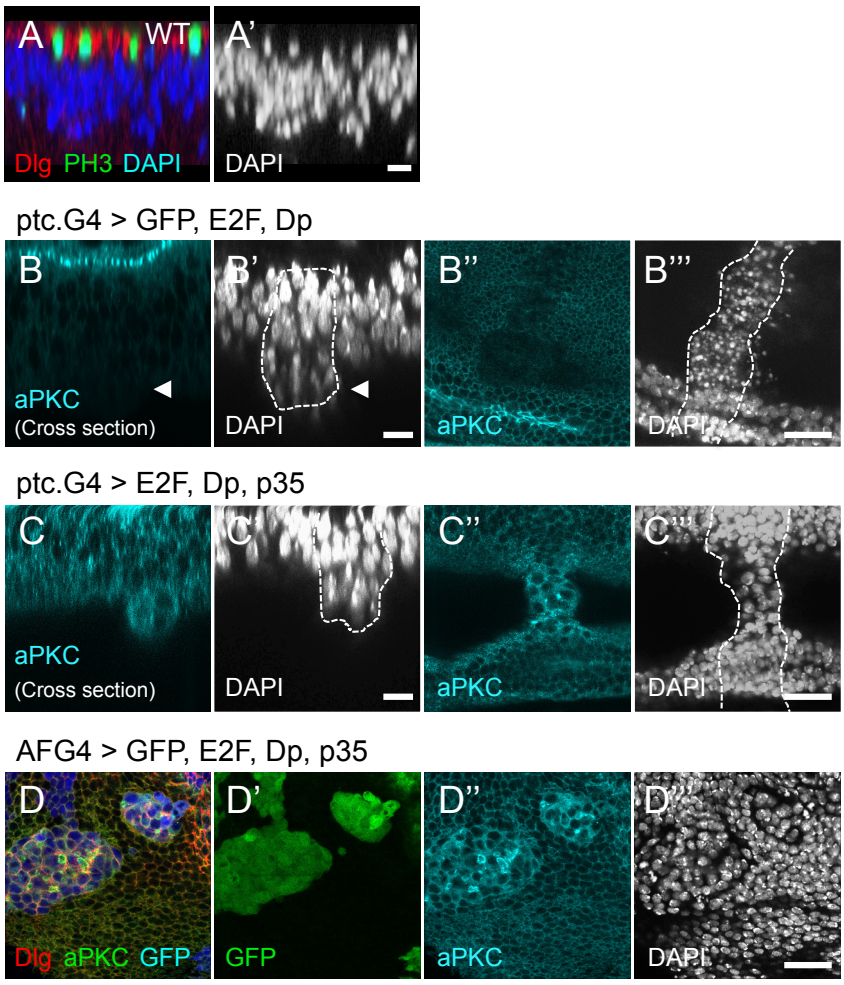

Figure 5.6 Forcing cell proliferation leads to elimination from epithelia and polarity deregulation

Mitotic cells are found near the apical surface of the wing disc epithelium.
Driving cell proliferation by expressing E2F and Dp under the ptcGal4 promoter causes cells to drop out from the epithelium (B', B''')
Driving cell proliferation and also expressing an anti-apoptotic factor causes cells to be eliminated from the epithelium (C'), but do not die, and show altered cell polarity (C'')
Forcing cell proliferation in flip-out clones also leads to perturbed cell polarity (D''). Scale bars 5μm (A, B-B', C-C'), 20μm (B''-B''', C''-C''', D).

5.4 Polarity, cyst formation and cancer progression

The causal link between polarity, cell proliferation and tumour development is fairly underdeveloped: to what extent does loss of polarity lead to tumours (e.g. similar to *lgl, scribble* and *dlg* mutants), and to what extent does some other mechanism lead to tumour formation and loss of polarity as a by-product (e.g. excess proliferation leading to cell elimination, and subsequent loss of polarity). Recently it was shown that spindle misorientation combined with anti-apoptotic factors could result in cyst-like development after cells escaped the epithelium (Nakajima et al., 2013). We also noted that excess proliferation combined with anti-apoptotic factors could lead to cells escaping the epithelium and displaying aberrant polarity. We were therefore interested to investigate polarity determinants in tumour development further.

We noted that stages in tumour progression could be seen in the development of colorectal cancer. Initially, non-invasive adenomas form, and by a series of steps including changes in expression of oncogenes and tumour suppressors, proceed to become adenocarcinomas and invasive carcinomas. Notably however, epithelial-mesenchymal transition (EMT), which is associated with cancer progression, only occurs in the final step, before which some adenocarcinomas still show the capacity to metastasise and lose epithelial polarity. The formation of cysts is also present in these stages. Typical models for colon cancer, like *APC* mutant mice expressing oncogenic Ras, do not fully recapitulate the phenotype as seen in human cancers. This is possibly due to the shorter lifespan of mice, which do not have time to acquire the multiple mutations required for metastasis and malignancy to develop. We therefore sought to use the simpler genetics of *Drosophila* to see if we could recapitulate the human-relevant phenotypes seen in cancer progression. Further details are available in our recently published review (Bell and Thompson, 2014).

Previously it was shown in cell culture that cells expressing oncogenic Ras are removed from a monolayer when surrounded by normal cells, but not other Ras-expressing cells: the boundary between normal and transformed cells is an important factor, rather than necessarily the particular state of the cell itself (Hogan et al., 2009). To investigate this *in vivo*, we expressed oncogenic Ras^{V12}

(Figure 5.7) or Arm^{S10} (data not shown) in the whole disc wing disc or in clones. Expression across the entire wing disc failed to show any severe phenotypes (Figure 5.7 A-B). We then made clones expressing Ras^{v12} or Arm^{S10}, and found that cyst-like structures formed (Figure 5.7). Interestingly, polarity seemed to be maintained in these cysts (Figure 5.7 D-E). We examined clones from different time points to try and assess the formation of these cysts. We noted that initially, there appears to be an accumulation of Myosin around the clone, and apical constriction and shortening of the junctions at the boundary of the clone and wild type cells (Figure 5.7 G). We suggest that this constriction continues, eventually leading to the clone forming a cyst.

Since the expression in the entire wing gave no real phenotype, we hypothesise that the interaction between wild type and oncogene-expressing clones leads to the elimination of these clones from the epithelium, where they can then form cysts outside of the epithelial layer. In addition to cell culture studies noted above (Hogan et al., 2009; Kajita et al., 2010), this is also reminiscent of studies in mice where *APC* mutant cells in the intestine are sorted out from their wild type neighbours (Barker et al., 2009).

Interestingly, the converse clone experiment, where the majority of the disc expresses Ras^{V12} and only a small portion is wild type, leads to the elimination of the wild type regions (A-K Classen, unpublished, and data not shown). Thus the interaction between the heterogeneous tissues seems to drive a cell-sorting process, rather than necessarily the nature of the oncogene itself, consistent with results from cell culture (Hogan et al., 2009; Kajita et al., 2010).

Although several oncogenes have been reported to form cysts, and we have investigated two (Arm^{S10} and Ras^{V12}), the expression of Yki-nls - the active form of the oncogene Yorkie shows a different effect. Clones expressing Yki-nls form small, spherical clones similar to Arm or Ras, but exhibit a different polarity localisation. In comparison to clones of Ras^{V12}, where polarity is somewhat regular and facing the centre of the cyst (e.g. aPKC is still apical relative to Dlg), Yki-nls clones show an inverted polarity, with enrichment of aPKC around the clone boundary (Figure A.9).

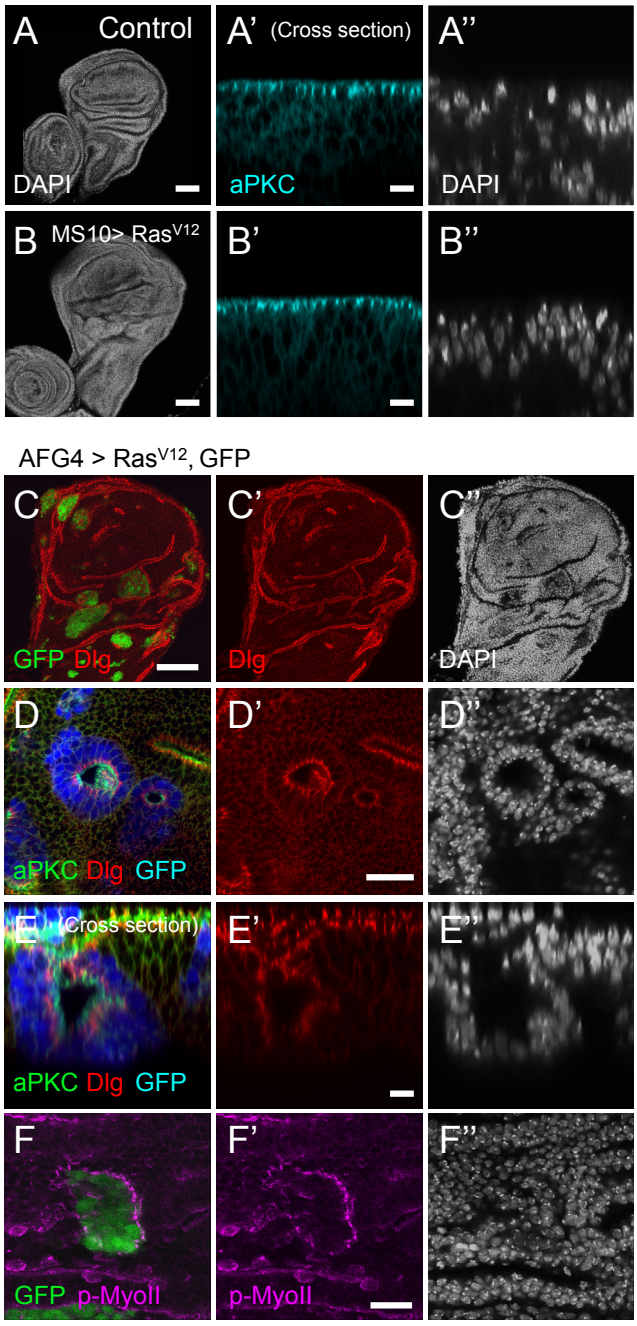

Figure 5.7 Development of cysts in wing disc epithelia

(A-B) Expression of oncogenic Ras^{V12} in the entire wing disc does not lead to gross morphology defects or loss of cell polarity compared to wild type (B).
(C-G) Expression of Ras^{V12} in clones results in small cysts forming in the wing disc. Note that polarity is still maintained in these cysts (D-E). Phospho-Myosin–II accumulates around the border of the Ras^{V12}-expressing clones (G). Scale bars 50μm (A, B, C), 5μm (A'-A'', B'-B'', E-E''), 20μm (D-D'', F-F'').

5.5 Summary

In addition to the changes to Lgl described in Chapters 3 and 4, we noted that adherens junctions components are down regulated during mitosis. This appears to be dependent on levels of Myosin-II, which is phosphorylated by Rho-kinase. Aurora kinases can phosphorylate Myosin-II, but this is likely to be necessary for cytokinesis, rather than cell rounding. The function of the adherens junction down regulation is not clear, and the extent of this phenomenon is variable between tissues. We found also that mitotic epithelia are more sensitive to mutations in polarity determinants than non-proliferating epithelia. Similarly, forcing cell proliferation resulted in cells being eliminated from the epithelium and losing cell polarity; though the initial driving force may be apical constriction due to cell over-crowding. Finally, we used *Drosophila* as a simple model for cancer progression. Activation of oncogenes is able to drive cyst formation, similar to colorectal cancer progression, and the heterogeneity of tissues seems to facilitate the sorting and removal of the minority genotype of cells. Collectively, these results emphasise the interesting dynamic between polarity and cancer, though much work remains in order to understand the links fully.

Chapter 6. Discussion

Our results suggest a new role for the polarity determinant Lgl in mitosis. The regulation of Lgl in both interphase and mitosis has also come under scrutiny, and we present a revised model in epithelial cells and asymmetrically dividing cells like neuroblasts.

6.1 Regulation of Cell Polarity

Mutual antagonism between apical and basolateral polarity determinants has long been thought as key for polarity establishment and maintenance, since the absence of one set of determinants leads to the spreading of the other. aPKC phosphorylates Lgl to restrict it to the basolateral membrane (Betschinger et al., 2003) (Betschinger et al., 2005), and Lgl is thought to reciprocally antagonise aPKC activity in an as-yet unknown mechanism (Yamanaka et al., 2003). Recently, it was shown that kinase-dead alleles of one of the key apical polarity determinants, aPKC, showed relatively mild phenotypes *in vivo* (Kim et al., 2009). Similarly in our work, *aPKC kinase-dead* clones were recovered easily in wing discs and did not show loss of polarity, in contrast to *aPKC null* mutant clones. The binding of aPKC to other apical polarity determinants and formation of a coherent apical complex, rather than necessarily the kinase-activity, may be a factor in epithelial cells. It has also recently been reported that Par-6 is an activator of aPKC activity (Graybill et al., 2012), and this may contribute to the necessity of an apical complex, rather than specific individual proteins, being important in polarity.

6.1.1 Expression of myrLgl does not affect cell polarity in epithelia

In our studies, we found that the basolateral polarity determinant and neoplastic tumour suppressor Lgl is relocalised in mitosis to the cytoplasm. In our efforts to understand the mitotic regulation, we used two constructs of Lgl, which in addition to failing to relocalise in mitosis, are also still present apically in interphase cells. Interestingly, neither the expression of myrLgl, which is tethered to the membrane all around the cell, nor Lgl3A, which cannot be phosphorylated by aPKC, disrupts cell polarity in epithelia, despite overlapping with aPKC. myrLgl might still be phosphorylated by aPKC at the apical membrane (and lose its inhibitory effect on aPKC), but remains there due to the myristoylation tag. This could be investigated

by checking for phospho-Lgl signal in *lgl* mutant clones rescued with myrLgl, or in Western blotting expressing myrLgl alone. Alternatively, the myristoylation tag could be interfering with Lgl's function in epithelia. Note however that myrLgl does seem to affect cell polarity in neuroblasts – Miranda is localised all around myrLgl-expressing cells, which suggests a reduction in aPKC kinase ability. Additionally, expression of myrLgl results in aPKC also being localised around the cell cortex (co-localising with Miranda and myrLgl). It is not clear why myrLgl has different effects in these different tissues. The presence of the Crumbs complex in epithelia might provide a stronger basis for maintaining cell polarity, through clustering and positive feedback of apical determinants (Fletcher et al., 2012). The *C. elegans* one-cell embryo is polarised and shows similar antagonistic interactions of polarity proteins to *Drosophila*: Par-6 and Par-2 are localised to the anterior and posterior respectively, and mutants for each lead to the spreading of the reciprocal domain (Goehring, 2014; St Johnston and Ahringer, 2010). However, the level of the polarity proteins, rather than simply their presence or absence, also affects domain size: *par-6* heterozygotes show an expansion of the posterior domain, without total loss of polarity (Goehring et al., 2011b). Like neuroblasts, *C. elegans* one-cell embryos represent a simpler polarised cell than epithelia, which have cell-cell junctions and additional polarity proteins (like the Crumbs complex). The different effects of expressing myrLgl (or Lgl3A) in neuroblasts or epithelia may therefore be a consequence of the relative complexities of these different cell types.

However, in epithelia, some constructs of various apical determinants are also able to spread laterally and co-localise with basolateral determinants, but do not obviously affect cell polarity: neither the expression of $aPKC^{CAAX}$, nor expression of $Crumbs^{FL}$ affects tissue morphology or cell polarity. Inhibition of endocytosis also results in apical and basal determinants overlapping (experiments performed by G. Fletcher).

Recently, it was shown that mutual antagonism alone is not sufficient for polarity generation in epithelia (Fletcher et al., 2012). In computer simulations, apical and basolateral determinants could also co-exist without disrupting cell polarity. These data suggest the view of polarity maintenance by mutual antagonism between apical and basolateral polarity determinants may be a little simplistic, and that the

contributions to polarity by the various determinants and their interactions should still be under careful consideration.

6.2 Localisation and regulation of Lgl in symmetric and asymmetric cell division

Cells appear to largely retain their polarity as they progress through mitosis, and most of the polarity determinants appear unchanged in wing disc epithelia. The major change we observed was the relocalisation of Lgl to the cytoplasm, and focussed on this phenomenon. However, some differences have been observed in different tissues. In follicle cells, aPKC and Crumbs were shown to be absent from the apical domain (Bergstralh et al., 2013b), and in the notum it is suggested that junctions may shift - and the apical domain extend - laterally (e.g. (Founounou et al., 2013) and A. Rosa, personal communication). The model of polarity in mitotic epithelia in Figure 6.2 focuses more on the role and regulation of Lgl, and presents a somewhat simplified version.

In epithelial cells, Lgl is thought to be phosphorylated by aPKC on a tripartite motif comprising serines 656, 660 and 664 (Betschinger et al., 2003). Phosphorylation of this motif then causes a conformational change in Lgl, resulting in auto-inhibition of Lgl's ability to bind the membrane, and thus phosphorylation results in a removal of Lgl from the membrane (Betschinger et al., 2005; Betschinger et al., 2003). We confirmed that the removal of Lgl from the apical membrane is indeed dependent on aPKC kinase activity, as non-phosphorylatable Lgl3A spreads apically, and wild type Lgl is apical in *aPKC kinase-dead* or *aPKC null* mutant clones. We confirmed that the mitotic relocalisation of Lgl is also phosphorylation dependent, as mutation of all three serines in the tripartite motif prevents relocalisation. Lgl has previously been reported as relocalising to the cytoplasm in epithelia (Huang et al., 2009), but neither the mechanisms nor reasons were characterised.

Much of the early work on the mitotic relocalisation of Lgl in the literature was performed in neuroblasts, where aPKC is polarised specifically in mitosis. Since aPKC is constitutively polarised, and presumably active, in epithelia, and we did not

observe any changes to aPKC localisation, we wondered whether aPKC was the kinase responsible for the complete relocalisation of Lgl during mitosis. Our characterisation was in wing disc epithelia, and it has recently been shown that aPKC (and Crumbs) are absent from the apical membrane in mitotic follicle cells (Bergstralh et al., 2013b). However, neither of these observations – aPKC being unaltered (our wing disc data) or absent (follicle cell data) – seems to support the role of aPKC in causing Lgl relocalisation, and we investigated this further. Interestingly, clones of *aPKC kinase-dead* or *aPKC null* mutants still showed Lgl relocalising in mitosis. An obvious candidate as the responsible kinase was Aurora A, which had previously been implicated in initiating the phosphorylation cascade resulting in Lgl relocalisation in neuroblasts (Wirtz-Peitz et al., 2008). The Aurora A consensus sequence matches the tripartite motif, and Aurora A shows similar mitotic localisation to a phospho-Lgl antibody. We showed that both Aurora A and B are both able to directly phosphorylate Lgl peptides, and suggest that this phosphorylation causes relocalisation of Lgl to the cytoplasm. Consistent with the Aurora consensus sequence, phosphorylation occurs only at serines 656 and 664 of the tripartite motif, and an LglASA construct with these two sites mutated to alanine remains cortical during mitosis. Notably, this construct is still removed from the apical membrane, presumably by aPKC kinase activity, and its expression in *lgl* mutant clones is able to fully rescue cell polarity. Thus, aPKC phosphorylates Lgl to restrict it basolaterally in polarity regulation, whereas Aurora phosphorylates Lgl to completely relocalise it into the cytoplasm during mitosis.

The differing functions of aPKC and Aurora in neuroblasts are hard to separate, because they are almost simultaneously activated in mitosis. In their paper proposing that aPKC is responsible for mitotic relocalisation of Lgl, Wirtz-Peitz and colleagues tested for direct phosphorylation of Lgl by Aurora A, and reported a negative result (Wirtz-Peitz et al., 2008). However, the phospho-Lgl antibody they used recognises only the middle serine in the tripartite motif, which does not match the Aurora consensus and is not an Aurora target (Wirtz-Peitz et al., 2008). The authors did note that Lgl is transiently polarised in mitosis (to the basal cortex) before its complete relocalisation, and our LglASA construct is localised basally but not in the cytoplasm. We propose that aPKC is responsible for the polarisation of

Lgl to the basal side of mitotic neuroblasts, and Aurora then phosphorylates Lgl to completely remove it from the cortex into the cytoplasm.

In neuroblasts, the phosphorylation and relocalisation of Lgl was proposed to be required to allow the introduction of Bazooka/Par-3 into the apical complex, which changes aPKC substrate specificity and results in phosphorylation and asymmetric localisation of cell fate determinants (Wirtz-Peitz et al., 2008). Whilst we offer a revision to their model in respect of the regulation of Lgl, the subsequent events remain valid, and an updated model is shown in Figure 6.2 A. Regulation is similar in symmetrically dividing cells: aPKC polarises Lgl basally, and Aurora phosphorylation directs the cytoplasmic relocalisation of Lgl in mitosis. Here, the distinction is clearer between the kinases, because aPKC is constitutively active and epithelial cells are also polarised in interphase. A model of mitosis in epithelial cells in shown in Figure 6.2 B.

6.3 Roles for Lgl in mitosis

6.3.1 Lgl in asymmetric cell division

In neuroblasts, altering levels or behaviour of Lgl affects aPKC activity, as implied by the localisation of Miranda, an aPKC-substrate and cell fate determinant. Phosphorylation of Lgl by aPKC polarises it to the basal cortex, away from apical aPKC, similar to epithelial cells. Lgl inhibits aPKC activity when the two are in a complex together (Wirtz-Peitz et al., 2008; Yamanaka et al., 2003), and thus must be removed to allow aPKC function. In *lgl* mutant clones, aPKC is allowed to spread around the membrane of the cell, rather than being restricted solely to the apical domain, similar to epithelia, though no clear explanation exists how *lgl* antagonises aPKC. This results in phosphorylation of Miranda all around the cell and its deposition into the cytoplasm. Consequently, without correct polarisation of cell fate determinants, *lgl* mutants show altered cell fate outcomes, with multiple neuroblasts being present in singly-generated clones.

The polarisation of Lgl to the basal cortex is critical to allow free aPKC activity, and expression of non-phosphorylatable Lgl3A, which is present around the entire cell

cortex during mitosis, results in mislocalisation of Miranda around the entire cell cortex (Figure 4.10). One suggestion for this is that Lgl3A has 'infinite buffering capacity' of phosphorylation by aPKC, and prevents aPKC from phosphorylating Miranda (Wirtz-Peitz et al., 2008). Similarly, expression of myrLgl, which is also present around the entire cell cortex, causes Miranda mislocalisation. Again, presumably the presence of myrLgl apically affects aPKC function and inhibits it, thus preventing aPKC from restricting Miranda to the basal side of the cell. Consistent with this, clones expressing myrLgl also show ectopic numbers of neuroblasts, implying faulty asymmetric division and daughter cell fate specification. The expression of myrLgl mimics Lgl3A in that aPKC's activity is presumably inhibited, given by the (mis)localisation of Miranda as a read-out. However, expression of myrLgl also results in aPKC spreading around the cell, which does not occur in Lgl3A cells, where it remains in an apical crescent. Neither does expression of myrLgl lead to spreading of aPKC in epithelia. It is not clear why myrLgl would lead to spreading (but inhibition) of aPKC. Although Lgl and aPKC can exist in a complex, before Lgl is phosphorylated and removed, this is proposed to be transient – hence the complementary localisations of Lgl and aPKC. Possibly the myristoylation tag affects some of the interactions between Lgl and aPKC: different effects in epithelia and neuroblasts might be due to the tissue specific differences noted previously.

The effects of expressing myrLgl or Lgl3A on cell polarity and Miranda localisation occur regardless of the presence or absence of endogenous Lgl – expression of constructs in *lgl* mutant clones shows similar results. Thus, in neuroblasts, the removal of Lgl from the apical membrane appears to be the critical factor. Our LglASA construct, which is polarised basally but does not relocalise to the cytoplasm, does not give any phenotype upon expression in either wild type or *lgl* mutant cells. The complete relocalisation of Lgl to the cytoplasm in neuroblasts therefore appears dispensable for correct asymmetric cell division.

6.3.2 Lgl in symmetric cell division

Unlike in neuroblasts, the expression of myrLgl or Lgl3A in wild type cells (i.e. with endogenous Lgl) did not appear to have any dominant effects in wing disc cells.

We therefore considered whether the relocalisation of Lgl in mitotic epithelia was necessary in order for Lgl to perform a specific role in the cytoplasm; as opposed to being removed from the membrane so as not to interfere with polarity. Interestingly, *lgl* mutant cells expressing either of our non-relocalising Lgl constructs, myrLgl or LglASA, showed defects in spindle orientation, with cell division no longer occurring in the plane of the epithelium. This mitotic role is distinct from its polarity functions, because *lgl* mutant cells expressing myrLgl or LglASA still retain cell polarity.

Recent studies have shown that both Dlg and Scribble, the two other members of the basolateral Scribble module, have roles in spindle orientation (Bergstralh et al., 2013b; Nakajima et al., 2013), with the role and mechanism of Dlg's action being better described. The mitotic spindle is oriented in part by Pins/LGN, which localises to the cell cortex and mitotic spindles, and binds to the microtubule-associated protein Mud/NUMA (Bergstralh et al., 2013b; Siller et al., 2006; Siller and Doe, 2009). Aurora A has been shown to phosphorylate Pins at serine 436 in its LINKER region in mitosis, which is essential for Pins to bind to the GUK domain of Dlg and orient the mitotic spindle (Johnston et al., 2009).

Interestingly, Pins is also reported as being excluded from the apical domain by aPKC (Guilgur et al., 2012), possibly through phosphorylation (Hao et al., 2010) and thus a dual regulation by aPKC/Aurora may occur, similar to what we found with Lgl. Phosphorylation by aPKC has been suggested to inhibit the binding of Pins to apical Gαi, and promote binding of Pins to 14-3-3 proteins, resulting in the exclusion of Pins from the apical cortex (Hao et al., 2010). In *aPKC* mutants, Pins would not be phosphorylated and excluded from the apical complex, and therefore would not be available for its role in spindle orientation (Guilgur et al., 2012). However, there are conflicting reports as to the ubiquity of this role for aPKC and the model of disrupted binding to Gαi is not fully satisfactory. For example: Pins has a wild type lateral localisation in *aPKC* mutant clones in follicle cells (Bergstralh et al., 2013a); aPKC is also absent from the apical domain of mitotic cells and would therefore seem unable to exclude Pins from this region; and aPKC is not required for planar spindle orientation in chick neuroepithelia, though Pins/LGN and Gαi are (Peyre et al., 2011). Overall, the data supporting a role for aPKC in spindle orientation are not convincing.

We found that the misorientation phenotype in *pins* mutants in the wing disc was weaker than in *mud* mutants or our *lgl*-rescue experiments. Previous papers on spindle orientation in the wing disc have not discussed *pins* phenotypes, or hinted that any effect is indeed mild (M. Gibson, unpublished; D. Bergstralh, personal communication). In follicle cells and neuroblasts however, *pins* mutants show stronger phenotypes, similar to *dlg* or *mud*. It is not clear why Pins appears somewhat dispensable for spindle orientation in wing discs. In a recent study, Banderuola was identified as a binding partner for Dlg and a novel regulator of spindle orientation in asymmetric cell division (Mauri et al., 2014). Bnd appears to act very upstream in some mitotic processes, since *bnd* mutants have mislocalised aPKC, Baz, Pins, Gai, Mud and Dlg in SOP cells, and spindle rotation is affected. *bnd* mutants however do not show fully penetrant phenotypes, and the authors suggest some partial redundancy. The full functions and interactions of Bnd are still unclear, but it is possible that it, or some as-yet-undiscovered protein, might play a role in some cell types but not others. The role – or lack thereof - of aPKC in spindle orientation also apparently depends on the tissue type (Bergstralh et al., 2013b; Guilgur et al., 2012), so a paradigm seems to exist for tissue specific roles, and Pins perhaps follows these examples.

mud is named for Mushroom Body Defects, due to the overgrowth phenotype seen in the mushroom bodies of the *Drosophila* brain (Technau and Heisenberg, 1982). Notably, *pins* mutants are not reported as showing overgrowth in this tissue. Whilst *mud* mutants display an increased number of neuroblasts, *pins* mutants actually have fewer neuroblasts (Lee et al., 2006b; Prokop and Technau, 1994). Additionally, whilst adult heterozygotes for *pins* mutant alleles or *mud* mutant alleles show reduced viability, *pins* heterozygotes are able to lay fertilised eggs, whereas *mud* flies are sterile (de Belle and Heisenberg, 1996; Yu et al., 2000).

Recently, novel roles for NuMA (the mammalian homologue of Mud) and dynein have been described at anaphase (Bergstralh and St Johnston, 2014; Kiyomitsu and Cheeseman, 2013; Kotak et al., 2013; Kotak and Gonczy, 2014; Seldin et al., 2013; Zheng et al., 2014). At anaphase, NuMA's localisation is independent of LGN/Pins, but may depend on Band 4.1, a cytoskeletal protein, or other

mechanisms (Kiyomitsu and Cheeseman, 2013; Zheng et al., 2014). NuMA is suggested to have a role in ensuring the spindle is centered in the cell, even if the spindle is misaligned (Kiyomitsu and Cheeseman, 2013), or in aiding in separating the spindle poles through pulling forces (Kotak et al., 2013; Zheng et al., 2014). Therefore, whilst Pins and Mud are binding partners involved in spindle orientation, they may have distinct in other processes and future work should consider this.

Recently it was shown that phosphorylation of Lgl on any of the serines in the tripartite motif allows Lgl to bind directly to Dlg *in vitro* (Zhu et al., 2014). Despite their strikingly similar phenotypes and localisations, this was the first evidence of direct binding of two members of the Scribble module. Importantly, phosphorylated Lgl binds to the same region of the Dlg GUK domain as does (phosphorylated) Pins, as revealed by crystal structures of the Dlg:Pins/LGN and Dlg:Lgl complexes (Zhu et al., 2014; Zhu et al., 2011a). We propose that the mitotic phosphorylation of Lgl by Aurora is required in order to remove Lgl from its binding to Dlg, and deposit Lgl in the cytoplasm. This then allows Pins, also phosphorylated by Aurora, to bind to Dlg and initiate spindle orientation. Subsequently, Dlg:Pins/LGN interacts with either Mud and Dynein/Dynactin, or Khc73, for microtubule shortening or microtubule attachment respectively (Johnston et al., 2009). Note however that the localisation of Lgl in interphase cells – all around the basolateral membrane - is slightly different from Dlg - which is enriched at the septate junctions - and therefore it is implausible that Lgl's basolateral localisation is completely due to its binding to Dlg. Rather than the pLgl:Dlg binding being a strict requirement for Lgl's basolateral interphase localisation, it may be that this interaction is key more for the regulation of Dlg's availability to bind to Pins, and thus orient the spindle. In a similar vein, note that LglASA is localised basally in mitotic neuroblasts, in contrast to Dlg's apical enrichment.

6.3.3 Symmetric vs. asymmetric cell division: similarities and disparities

Although the phosphorylation-dependent regulation of Lgl is the same in both tissues – polarisation by aPKC and cytoplasmic relocalisation by Aurora – Lgl has distinct roles in mitosis in neuroblasts and epithelia, as revealed by the use of our LglASA construct. Why then is the polarity function of Lgl required in neuroblasts

(but not epithelia), and the spindle orientation function required in epithelia (but not neuroblasts)? One solution is that neuroblasts have an additional factor in orienting the spindle via the activity of Inscuteable, which is not expressed in epithelial cells. Inscuteable forms an apical crescent in mitosis, and recruits Pins to the apical membrane where it (Pins) is bound by Gαi proteins to the cortex. Pins:Insc binding is then released, and Pins is free to bind to Mud or Dlg, to orient the spindle (Johnston et al., 2009; Siegrist and Doe, 2005).

As discussed, polarity in epithelia is fairly robust, and the abrogation of aPKC kinase activity is not sufficient to greatly disrupt cell polarity (Kim et al., 2009). Unlike neuroblasts, epithelia are constitutively polarised and also express Crumbs, an apical polarity determinant involved in the maintenance of cell polarity (Fletcher et al., 2012). Thus it appears that in neuroblasts, Lgl is required as a player in polarity, helping to regulate aPKC activity; but is not needed for spindle orientation due to the presence of Inscuteable. In epithelia, where polarity is more robust and is largely maintained during mitosis; Lgl is free to participate in regulation of spindle orientation, which may need an additional regulator compared to neuroblasts since Inscuteable is not expressed.

In our model, the presence of Lgl bound to Dlg prevents the formation of the Dlg:Pins complex needed for spindle orientation. One might therefore expect there to be a dominant effect if either LglASA or myrLgl are expressed in a wild type background, similar to neuroblasts. However, we only saw spindle orientation defects when expressing LglASA or myrLgl in *lgl* mutant clones. We suggest that in *lgl* mutant cells expressing LglASA – such that there is no other form of Lgl in the cell – only LglASA is present to bind to Dlg. Then, in mitosis, LglASA is not relocalised to the cytoplasm, because Aurora cannot phosphorylate it. The continued presence of LglASA bound to Dlg therefore prevents Pins binding to Dlg, resulting in the disruption of the spindle orientation pathway. In a wild type background, the endogenous Lgl (as well as LglASA) can bind to Dlg. Upon phosphorylation by Aurora in mitosis, endogenous Lgl will be relocalised to the cytoplasm, allowing Pins – or other Lgl molecules – to bind to the now-available Dlg. The binding affinity of Pins with Dlg is greater than Lgl with Dlg (Zhu et al., 2014; Zhu et al., 2011a), so upon the removal of Lgl by Aurora, it is likely that Pins would

preferentially bind to Dlg, and thus aid in spindle orientation. Finally we note that spindle misorientation phenotypes in the wing disc tend to be relatively mild (Nakajima et al., 2013), and the masking effect we suggest, in addition to the rarer phenotypes seen, may lead to unobservable defects. Validation of *in vitro* findings (such as the pLgl:Dlg binding), and comprehensive analysis of binding interactions and competition should shed light on this area.

6.3.4 Phosphorylation of Lgl by aPKC: implications from our model

aPKC has been shown to be able to phosphorylate all three residues in the Lgl tripartite motif *in vitro*, and phosphorylation of Lgl is required for it to bind to Dlg at the lateral membrane. We have shown that Aurora phosphorylates serines 656 and 664 of the same tripartite motif to relocalise Lgl into the cytoplasm. This then raises the question: how can Aurora phosphorylate those serines, if they have already been phosphorylated to exclude Lgl from the apical membrane? The same question can be asked of Pins, which is supposedly phosphorylated by both aPKC and Aurora at serine 436. One possibility is the action of a phosphatase. Since the binding of Lgl to Dlg is dependent on the phosphorylation of Lgl, a phosphatase would probably have to act specifically on S656 and S664: thereby allowing Aurora to regulate Lgl during mitosis. We observe that after mitosis, Lgl returns to a cortical localisation: the existence of a phosphatase would also provide an explanation for this effect.

An alternative explanation is that aPKC only phosphorylates Lgl on the middle serine, S660. We have shown that LglASA, where only S660 is available for phosphorylation by aPKC, is still completely excluded from the apical membrane. Moreover, cells only expressing LglASA – i.e. in an *lgl* mutant background – retain their polarity. Phosphorylation of S660 by aPKC is therefore sufficient for apical exclusion and the polarity functions of Lgl. aPKC may be able to phosphorylate the other serine residues *in vitro*, but perhaps not for functional importance *in vivo*. Consistently, the recent paper showing that Dlg and Lgl bind proposes that phosphorylation of any one of the three serines results in the ability of phospho-Lgl to bind Dlg; and that peptides of double-mutant Lgl (e.g. LglASA, LglSAA, LglAAS) each are able to bind with comparable strength as wild type (LglSSS).

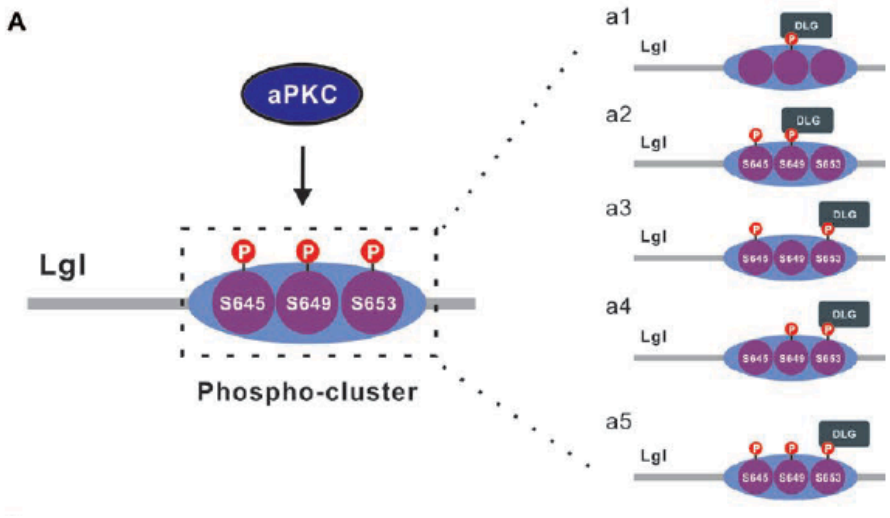

Figure 6.1 Model of phosphorylation of Lgl

Figure from (Zhu et al., 2014). Due to biochemical and stoichiometric limitations, there are five possible patterns of phosphorylation of Lgl by aPKC. Dlg can only bind to one phospho-serine in each case. Note that serines here are numbered in accordance with Lgl2.

Thus, although *in vitro* aPKC might appear to phosphorylate all three of the serines with some variability, phosphorylation of a single site is sufficient to bind to Dlg. Which serine or serines are actually responsible for Dlg:Lgl binding? The authors of the binding study suggest that careful analysis of the crystal structures and biochemistry may aid in answering this question. They propose that there are five possible phosphorylation patterns for the tripartite motif: in each case, Dlg is able to only bind to one phospho-serine, and that this is the most C-terminal phospho-serine (Zhu et al., 2014)(Figure 6.1). The authors therefore rule out the possibility that aPKC phosphorylates Lgl on only S656, or only S664. Although they consider the possibility of aPKC phosphorylating all three serines (Figure 6.1 'a5'), or just S656 and S664 (Figure 6.1 'a3'), both of these patterns would mean that Aurora is unable to further phosphorylate Lgl, which does not fit our data without considering the presence of a phosphatase.

Use of various Lgl constructs could provide insight into exactly which serines are phosphorylated by aPKC. If Zhu and colleagues' 'a2' scenario is correct (Figure 6.1) and aPKC phosphorylates S656 and S660, an LglSSA construct would localise laterally and bind to Dlg. However, in mitosis, Aurora would be unable to

phosphorylate LglSSA, since S656 is already phosphorylated (by aPKC) and S660 is not an Aurora target site. LglSSA would therefore not relocalise to the cytoplasm during mitosis. In contrast, LglASS would localise laterally and bind to Dlg, and would also be relocalised in mitosis, since Aurora could phosphorylate S664.

6.3.5 Other questions from the model

Although binding of Dlg:Pins/LGN is proposed as being stronger than Dlg:Lgl (Zhu et al., 2014; Zhu et al., 2011a), no direct competitive assays have been performed. It would be interesting to test this. Our results showed that expression of LglASA does not affect spindle orientation, and we suggest that this may be due to a temporal loophole after Aurora has removed endogenous Lgl at mitosis, leaving Dlg available for binding by either Pins or Lgl. In contrast when only LglASA is present in the cell, there is no window for Pins to bind. We could look to see whether the ability of Dlg to bind to Pins is altered by the co-expression of various Lgl constructs. For example, expressing Pins, Dlg and Lgl with aPKC – to set up the initial conformation – and then adding Aurora to replicate mitosis. Wild type Lgl would bind to Dlg, but then be displaced (by Aurora phosphorylation), and therefore not affect Dlg-Pins binding. Similarly, expression of Lgl3A should not disrupt Dlg-Pins binding, since Lgl3A cannot be phosphorylated and so cannot bind to Dlg initially. In contrast, LglASA would bind to Dlg and then prevent Dlg from binding to Pins (since it cannot be relocalised by Aurora).

It should also be noted that our model is based on research from several slightly different systems, and therefore confirmatory work would ideally be required. For example, the binding of Dlg to p-Lgl was performed in cell culture and based on structural analysis, and it will be interesting to see whether similar results can be achieved *in vivo*. Differences have already been mentioned earlier in this thesis between wing disc and follicle cell epithelia: for example, the localisation of determinants in mitosis, the extent of spindle misorientation for members involved in orientation, and the possible consequences of cells undergoing misoriented mitoses. Care should therefore be taken to not simplify too much, and clarify the extent of generalised observations within more specific tissue and model types. For instance, misoriented spindles resulting from mutations in *pins*, *mud* and *dlg*

appear less frequently in wing discs than follicle cells. One explanation may be that there is a tendency for the elimination of daughter cells (from misoriented mitotic events) from the epithelium in wing discs; whereas daughter cells initially divided out of the plane of the epithelium can sometimes reinsert themselves into the epithelial layer in follicle cells.

In summary, LglASA - where only S660 is able to be phosphorylated - is excluded from the apical region, and *lgl* mutants expressing this construct exhibit normal polarity: phosphorylation of S660 alone is sufficient for polarity maintenance. Phosphorylation of any one of the three serines in the tripartite motif allows Dlg to bind Lgl with comparable strength to wild type, and Dlg can in fact only bind to one phospho-serine, regardless of the pattern of phosphorylation. We feel this provides a reasonable basis for speculating that aPKC phosphorylates only S660 *in vivo*, leaving S656 and S664 available for phosphorylation by Aurora for the mitotic relocalisation.

6.3.6 A role for Lgl in spindle assembly?

Previous work has suggested that Lgl2, a human homologue of Lgl, has a role in spindle formation in mammalian cell culture (Yasumi et al., 2005). The authors report that Lgl2 binds directly to LGN strengthening the physical interaction between LGN and NUMA (*Drosophila* Pins and Mud). Knockdown of *lgl2* results in aberrant spindle formation, including monopolar and multipolar spindles (Yasumi et al., 2005). Spindle formation defects have also been reported in *aurora A* mutants, in addition to the expected centrosome and mitotic phenotypes (Berdnik and Knoblich, 2002). We found that *lgl* mutant discs showed many misfired spindles, including monopolar spindles. Importantly, this was not a general loss-of-polarity phenotype, because *dlg* mutant discs displayed regular spindle formation. We observed mitotic spindles misoriented with respect to the plane of the epithelium in both *lgl* and *dlg* discs, but due to the loss of polarity, could not comment on any misorientation effects in this experiments. Similar effects were observed by live imaging. Although the spindle misorientation phenotype in *pins* mutants is well established (at least in some cell types) (Bergstralh et al., 2013b; Izumi et al., 2006), a role for spindle formation appears less clear. Knockdown of LGN can cause

spindle disorganisation in mammalian cells (Du et al., 2001; Yasumi et al., 2005), but neither in published work (Bergstralh et al., 2013b) nor our own experiments did we note spindle disorganisation in *pins* mutants. It is therefore not clear, nor does it seem satisfyingly plausible, that the defects seen in *lgl* discs are strictly due to defects in the formation of the Pins:Mud complex. However, Pins is also present at the spindle and not just the cortex in mitotic cells (Bergstralh et al., 2013b), and our observations of spindle formation defects (in *lgl* mutants) are supported by other groups' results (E. Morais-de-Sa, unpublished). We therefore must consider that the importance of Lgl in mitosis may not only be to help regulate spindle orientation (by its timely removal from the membrane), but that the relocalisation to the cytoplasm is also a meaningful event in allowing Lgl to contribute to spindle formation. This area is still under-developed, and more work will be necessary to clarify this potential function of Lgl.

Detailed observations from live-imaging of large numbers of mitotic cells in *lgl* mutant discs would be useful to quantify spindle assembly and timing defects: in this work we noted some effects in live-imaging but not enough to quantify effects, nor we did not live-image *dlg* mutant discs as a control. This analysis could also be performed in *Drosophila* S2 cells, where the relative ease of imaging would assist in generating a large data set. Comparison of wild type, *lgl* mutant, and any other interesting candidates (*pins*, *mud; dlg* for control) would then help clarify any effects we noted. For instance: are spindle/microtubules the same size in *lgl* mutants as wild-type (Figure 4.4 B might suggest a difference); what proportion of cells show spindle assembly defects; what proportion of these cells which appear to proceed through mitosis slow but without chromosome segregation problems, and what proportion result in lagging chromosomes (as in Figure 4.4 C). The relationship of a mitotic cell's environment, its size, and the forces upon it have some effect on spindle morphology (Cadart et al., 2014; Lancaster and Baum, 2014; Lancaster et al., 2013), and a disorganised *lgl* mutant wing disc might not be the best environment for cells to divide. Imaging of *dlg* mutant discs or *lgl* knockdown in S2 cells could help address this issue too.

6.4 Redundancy of Aurora A and B

In elucidating the kinase responsible for phosphorylating Lgl in mitosis, our attention was initially focussed on Aurora A. We observed that *aurora A* mutant wing discs, or discs expressing Aurora A RNAi, showed strong delays in the relocalisation of Lgl, though it does eventually occur. The Aurora A and B consensus sequences are closely matched, and both kinases are able to phosphorylate some of the same targets (Carmena et al., 2009; Kunitoku et al., 2003; Zeitlin et al., 2001). Additionally, although the main functions and localisations of Aurora A and B are distinct, relating to the centrosome and kinetochore for A and B respectively, both kinases do exhibit dynamic localisations and cytoplasmic pools (Berdnik and Knoblich, 2002; Carmena and Earnshaw, 2003; Fu et al., 2007; Murata-Hori et al., 2002).

On inactivating both kinases using the VX-680 drug, we no longer observed Lgl relocalisation, and could not detect phospho-Lgl in Western Blotting. We were mindful that depleting important cell cycle kinases might lead to indirect effects, rather direct phosphorylation events: the depletion of Aurora A in *Drosophila* or mammalian cells leads to mitotic delays (Du and Hannon, 2004; Dutertre et al., 2002; Lee et al., 2006a) and this might explain the slow release of Lgl into the cytoplasm. Although treatment of discs with VX-680 greatly reduced the mitotic index, occasional escapers were found; more prominently on the removal and eventual wearing off of the drug. We are confident these cells are mitotic because they still round up and undergo interkinetic nuclear migration, hallmarks of mitosis (Meyer et al., 2011; Spear and Erickson, 2012; Thery and Bornens, 2008). However, the direct kinases assays were still critical to perform, and these backed up the idea that both Aurora A and B could directly phosphorylate Lgl. In addition, both kinases could phosphorylate CENP-A, used as a positive control, and to a slightly lesser extent, Sqh (MRLC), highlighting the potential redundancy between the kinases.

Our analysis of clones or regions of Aurora A, Aurora B, and double Aurora A/B depleted cells supports our data from the drug treatment that both kinases are required for efficient mitotic entry. Mammalian cells treated with a different dual

kinase inhibitor (ZM4473) did continue to enter mitosis after addition of the drug (Van Horn et al., 2010), and we found that varying concentrations and drug-addition times resulted in a spectrum of effects. Feedback loops involving both Aurora A and B have been proposed to aid in the control of cell cycle progression (Van Horn et al., 2010; Wang and Zhou, 2012; Wu et al., 2012; Zimniak et al., 2012), and it seems likely that the redundancy between the Aurora kinases is also dependent on temporal and dosage regulation.

As yet, relatively little is known about the extent of the redundancy and key substrates remain to be elucidated (Hochegger et al., 2013). Our results add to the limited but growing collection of work addressing Aurora functions, and suggest Lgl as an important target. Additionally, both Aurora A and B are implicated in tumour formation and progression, and work to target the Auroras in treating cancer should take into account the redundancy between the kinases (Giet et al., 2005; Marumoto et al., 2005; Marzo and Naval, 2013; Sausville, 2004).

6.5 Polarity, mitosis and cancer

We observed that apical-basal polarity is maintained during mitosis, with most polarity determinants unaltered through the cell cycle (Guillot and Lecuit, 2013). In addition to the dramatic changes to Lgl, we also noted that adherens junctions components are down regulated in wing disc cells, and that this is dependent on Rho-kinase activity. It had previously been reported that Armadillo/β-catenin levels are reduced in mitotic cells, but this observation was loosely speculated as having a role in Wnt signalling (Marygold and Vincent, 2003). We noted that ECadherin levels were reduced too, and contemplate that this down regulation may be required to accommodate the shape changes that occur as mitotic cells undergo interkinetic nuclear migration and cause local tissue deformation. The extent of this deformation is dependent on the changes between interphase and mitotic morphology: since interphase wing disc cells are tall, thin and pseudostratified, and the shape changes between interphase and mitotic wing disc cells is so dramatic, it may be that other tissues do not require the down regulation that we observed here (Founounou et al., 2013; Guillot and Lecuit, 2013).

Recent work has suggested that cells dividing out of the plane of the epithelium due to spindle orientation defects can form tumour-like growths if prevented from undergoing apoptosis (Nakajima et al., 2013). We observed spindle orientation defects on altering Lgl function, and it would be interesting to express anti-apoptotic factors in these experiments, to see whether we observe tumour-like growths similar to those proposed for *mud* or *rok* depletion (Nakajima et al., 2013). In a similar vein, we found that forcing cells to proliferate rapidly caused elimination from the epithelium, and these cells showed polarity defects if kept alive by the co-expression of cell survival factors. However, it is not clear that cell division out of the plane of the epithelium is a key factor in EMT-like effects. In other tissues, cells dividing out of the plane of the epithelium are sometimes able to re-establish themselves (D. Bergstralh and D. St Johnston, unpublished). Despite cell rounding and interkinetic migration resulting in cells at the apical membrane, mitotic cells are likely to retain some contact with the basement membrane, perhaps through retraction fibres (Meyer et al., 2011). Cells do not therefore lose complete association with their position in the epithelium, and even if they divide misaligned to the epithelial layer, they may still be able to reassert themselves.

Drosophila can also serve as a model for cancer progression complementary to well-used mouse models. We used the simpler genetics of *Drosophila* to investigate cell and tissue changes similar to those seen in colorectal cancer progression. Prior to full-blown metastatic carcinomas, expression of certain genes can cause cysts to form: these may retain epithelial character and polarity, but form outside the epithelial layer. The heterogeneity of the tissue itself may provide a basis for cell sorting and the segregation of any minority of cells into cyst-like structures (Barker et al., 2009; Bell and Thompson, 2014; Hogan et al., 2009; Kajita et al., 2010). After more than a century of research, the techniques and insights provided by *Drosophila* continue to provide ample opportunity to dissect molecular bases for cancer development and progression.

6.6 Open Questions

From the experiments performed during the course of the research comprising this thesis, a number of questions have arisen which remain unanswered and deserve future consideration. These have been noted and discussed throughout the thesis, and are compiled here.

6.6.1 aPKC-mediated phosphorylation of Lgl, and binding to Dlg

Recent work suggests that phosphorylated Lgl binds to Dlg. However, this cannot be the sole reason why Lgl is localised basolaterally, since the localisation of Lgl is broader than that of Dlg, which is enriched at septate junctions. Lgl is also localised normally in clones of cells mutant for the DlgGUK domain, where pLgl supposedly binds (D. St Johnston, personal communication). How is 'trespassing' Lgl at the apical membrane restricted basolaterally, and what it then binds to at the basolateral membrane? Note that the pLgl antibody detects signal at the apical domain of cells (where Lgl is phosphorylated by aPKC), but not basolaterally. In neuroblasts, LglASA is localised basally, whereas Dlg is enriched apically: pLgl:Dlg binding in this case seems unlikely. How is LglASA localised? (See Chapter 6.1)

In our model, a pLgl:Dlg interaction is replaced, upon the phosphorylation and removal of Lgl, by Pins:Dlg. Why does expression of non-phosphorylatable LglASA not show a dominant phenotype in blocking Pins:Dlg binding? This may be due to the presence of endogenous Lgl in the cell - which can be removed from Dlg at mitosis – and subsequent competition between Pins and Lgl for Dlg, but this is currently unclear. (See Chapter 6.3.3).

6.6.2 myrLgl

In neuroblasts, expression of LglASA in wild type or *lgl* mutant cells does not affect spindle orientation: this is probably because LglASA is polarised to the basal domain, whereas Dlg (and Pins) are apical. myrLgl however is localised all around the membrane. Why does expression of myrLgl not affect spindle orientation? (See Chapter 6.3.1)

Expression of myrLgl in epithelia does not affect cell polarity, despite localising with aPKC at the apical domain. Is myrLgl still phosphorylated (and 'inactive' in its inhibition of aPKC)? Does the myristoylation-tag affect its activity?
Expression of myrLgl in neuroblasts does affect cell polarity, as determined by Miranda localisation. Why does it disrupt polarity in neuroblasts but not epithelia? Similarly, why does expression of myrLgl cause aPKC to spread around the membrane in neuroblasts? (See Chapter 6.1)

6.6.3 Lgl in spindle assembly

Lgl appears to play a role in assembling the mitotic spindle. How does Lgl perform this function? A role for Pins seems unlikely, in contrast to mammalian LGN in cultured cells. What other players could be involved with Lgl here? Pins itself may be mislocalised in *lgl* mutant discs: why is this? Is the slower speed of mitosis phenotype that is seen in (some) *lgl* mutant cells a consequence of this spindle assembly defect? What proportion of *lgl* mutant mitotic cells show spindle defects and/or slower speeds through mitosis? (See Chapter 6.3.6).

6.6.4 Spindle orientation: tissue differences and unresolved questions

In follicle cells and neuroblasts, Pins, Mud and Dlg have well-established roles in spindle orientation. In wing discs, Pins appears somewhat dispensable for metaphase spindle orientation, whilst Mud is still important. Recent evidence suggests novel roles for Mud/NuMA in anaphase, distinct from any interaction with Pins. What is Mud/NuMA doing in anaphase (spindle pulling? Spindle centering?), and how does it mediate this independent of Pins? Does Mud operate in a separate pathway from Pins in wing discs to orient that spindle? Novel players in spindle orientation are still being discovered and it would be prudent to maintain an open mind with regards existing models. (See Chapter 6.3.2)

6.6.5 General questions

Why do cells divide at the apical region of tissues (notably in the wing disc, in this thesis)? Is it because the players for accurate mitosis are there – e.g. Dlg, Mud for spindle orientation; Mud for spindle pulling (see above)? Most polarity proteins and junctions are also localised to the apical surface.

aPKC and Crumbs show different localisations in mitosis in follicle cells and wing discs. Dynamics of adherens junctions (e.g. down regulation) appear variable in different tissue types (wing vs. notum). aPKC is reported as having a role in spindle orientation in the wing disc, though the data is unconvincing, but not in follicle cells or chick neuroepithelia. myrLgl appears to behave differently in epithelia and neuroblasts. Care should therefore be taken to consider tissue-specific effects, even if the core proteins appear similar.

6.7 Summary

Our findings suggest a revised model for the regulation and function of Lgl in both symmetric and asymmetric divisions. In symmetric divisions, we have uncovered a novel function for Lgl in aiding the orientation of the mitotic spindle. In our model, Lgl is phosphorylated by aPKC and restricted basally, where it binds to Dlg. In mitosis, Aurora A then phosphorylates Lgl to relocalise it to the cytoplasm; allowing Pins to bind Dlg and initiate spindle orientation. Lgl may also have a role in spindle formation. In neuroblasts, Lgl is dispensable for spindle orientation but instead is required to help control polarity, through its interactions with aPKC. Phosphorylation of Lgl restricts (by aPKC) it to the basal cortex, thus limiting aPKC activity to the apical domain. aPKC is then able to act to generate asymmetric localisation of cell fate determinants. Spindle misorientation has been linked to the development of tumour-like growths, and the identification of Lgl as a player in this process should be considered in future work on Lgl's role as a tumour suppressor.

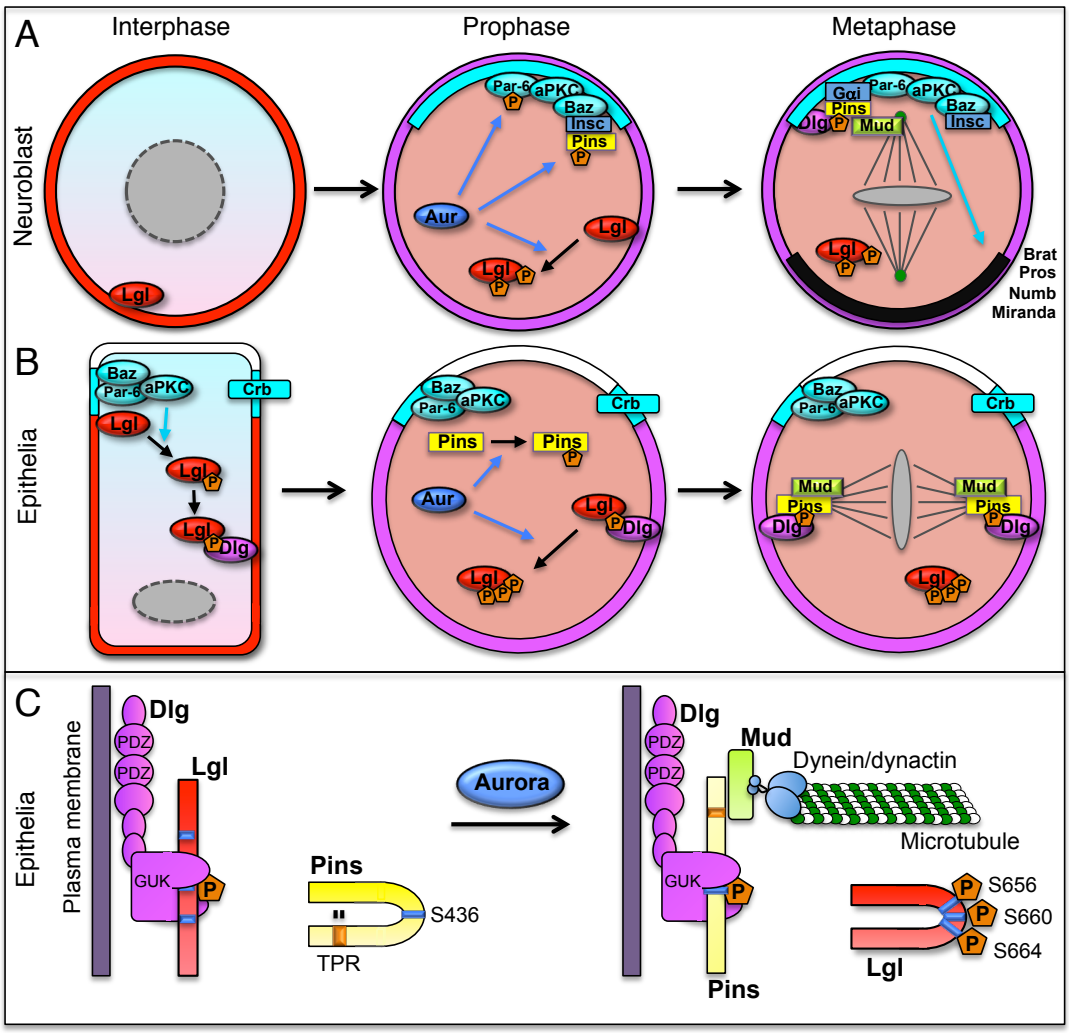

Figure 6.2 Models of mitosis in neuroblasts and epithelia

(A) Mitosis in neuroblasts. Aurora phosphorylates Lgl in mitosis, causing Lgl to relocalise to the cytoplasm. Phosphorylation of Par-6 and Pins by Aurora are required for correct segregation of cell fate determinants and spindle orientation respectively.
(B-C) Mitosis in epithelial cells. aPKC-phosphorylated Lgl binds to Dlg at the basolateral membrane in interphase. At mitosis, Aurora phosphorylates Lgl on S656 and S664 to relocalise Lgl to the cytoplasm. Aurora also phosphorylates Pins at S436. These events allow for the formation of a Dlg:Pins complex, facilitating spindle orientation.

Appendix

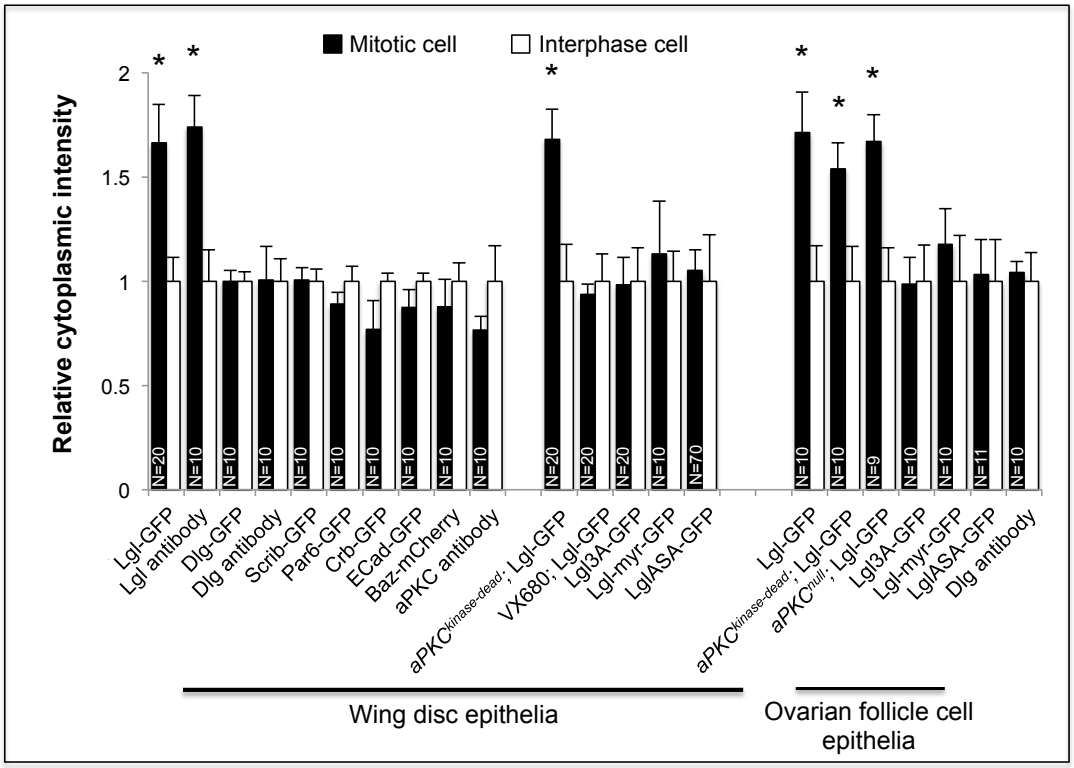

Figure A.1 Quantification of mitotic changes in polarity determinants

Statistical analysis of mitotic vs. interphase cells was performed using a two-tailed t test. An asterisk denotes a p value < 0.05.

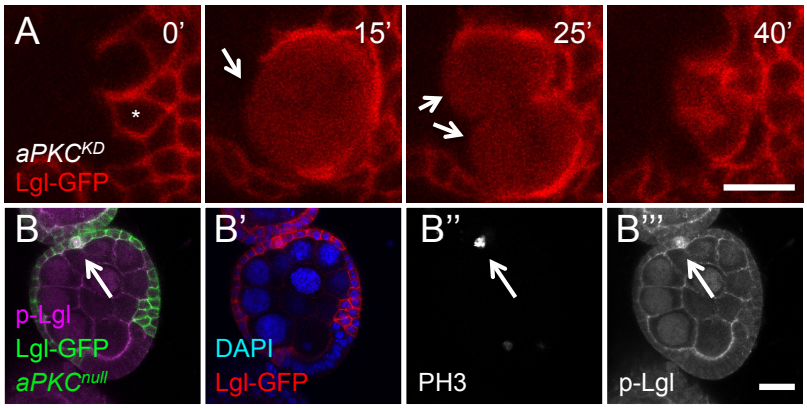

Figure A.2 Lgl mitotic relocalisation is aPKC-independent

(A) Lgl-GFP still relocalises to the cytoplasm in mitotic cells of *aPKC*$^{kinase\text{-}dead}$ clones in the wing disc. Note that Lgl is completely absent from the cortex in the region on the edge of the clone (15' and 25'). The brighter crescents around the mitotic cell in 15' and 25' are due to neighbouring cells distorted due to the mitotic cell rounding. Clones of the *aPKC*^{psu265} allele are shown.
(B) Phospho-Lgl antibody still accumulates in mitotic cells of *aPKC*^{null} mutant clones in the follicle cell epithelium, marked by Lgl-GFP. Note the absence of interphase Phospho-Lgl staining in the clones. Lgl-GFP also is still relocalised to the cytoplasm in the mitotic cell. Scale bars 5μm (A), 20μm (B).

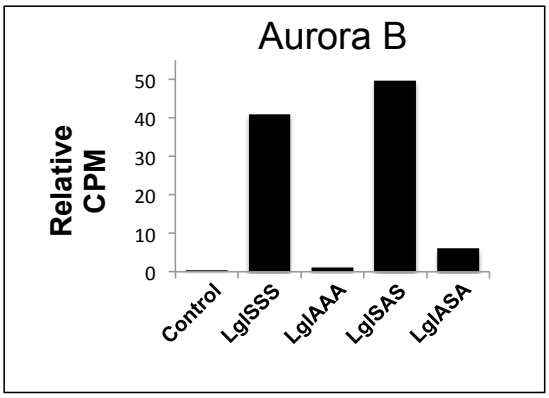

Figure A.3 Aurora B phosphorylates Lgl on S656 and S664

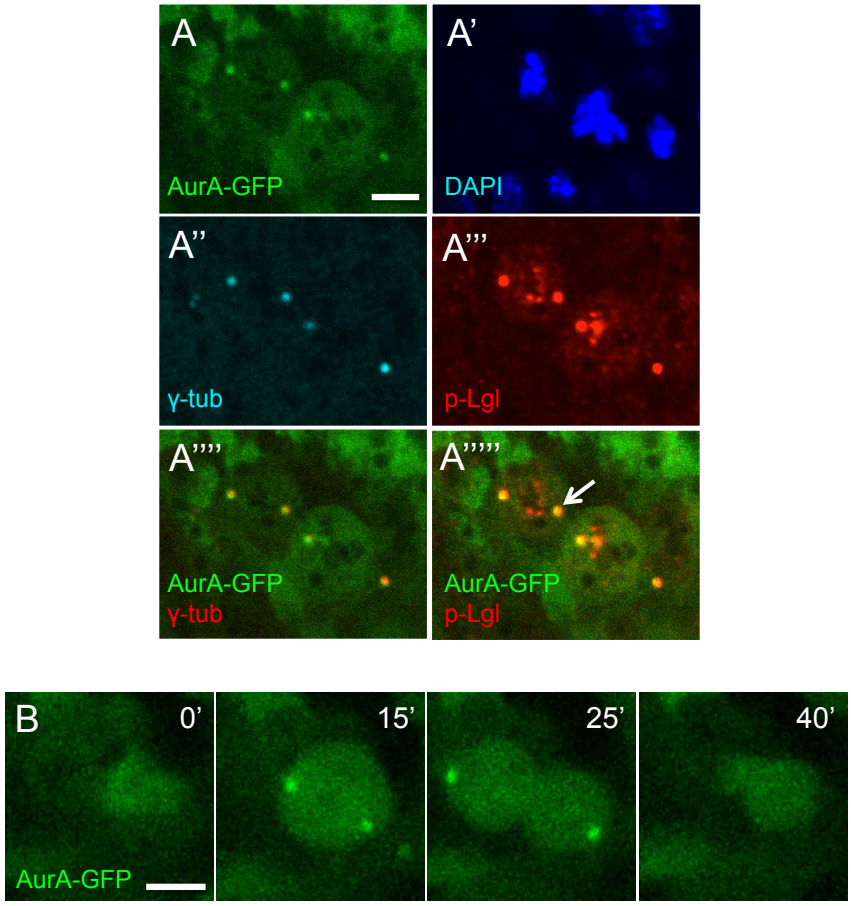

Figure A.4 Aurora A-GFP and phospho-Lgl accumulate at centrosomes

(A) Aurora A-GFP, phospho-Lgl antibody and γ-tubulin accumulate and co-localise at centrosomes.
(B) Live imaging of Aurora A-GFP in wing discs. Note that cytoplasmic pool of Aurora A, as well as centrosomal enrichment. These pools are dynamic and interchangeable (Berdnik and Knoblich, 2002). Scale bars 5μm.

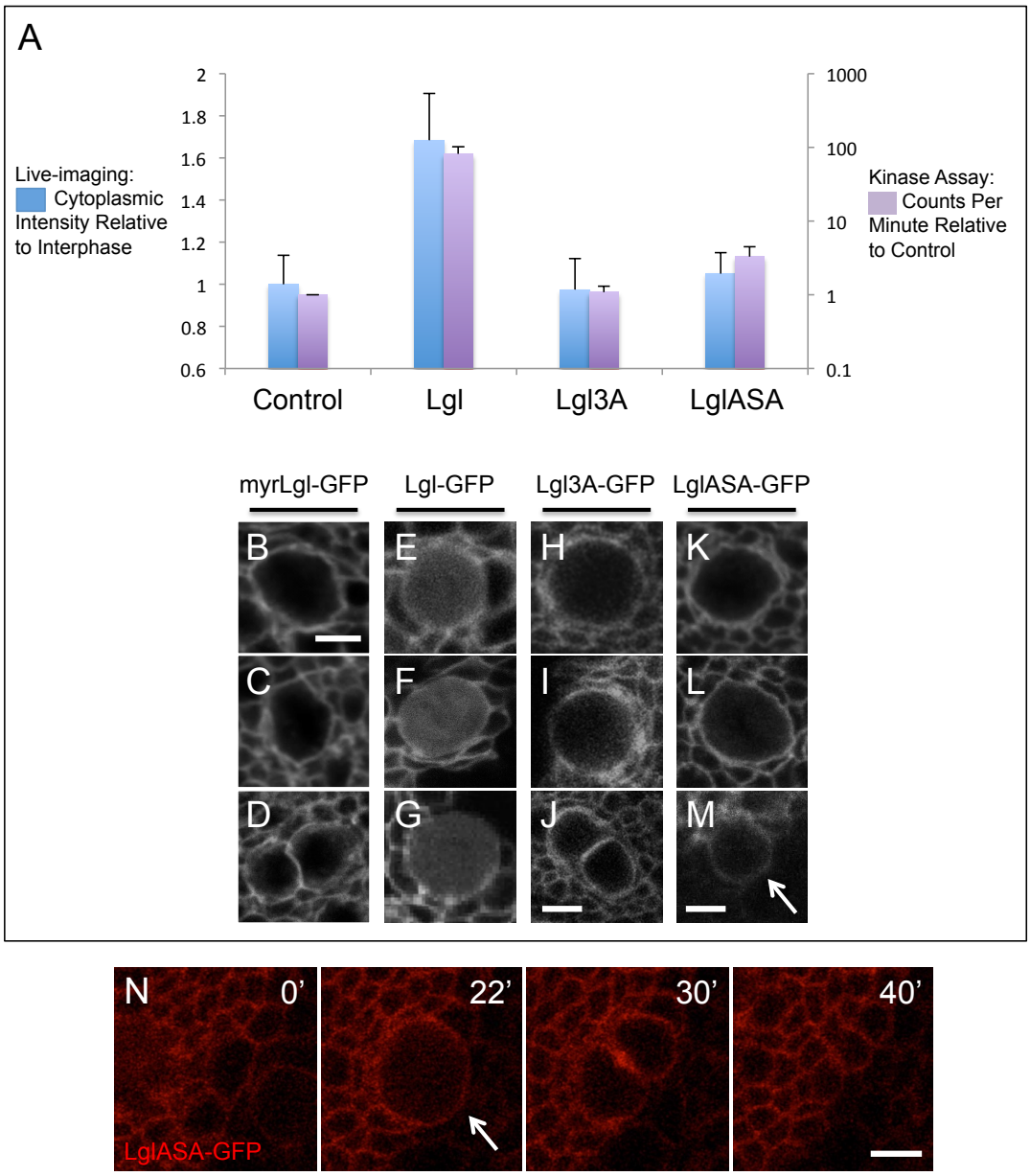

Figure A.5 Behaviour of Lgl constructs

(A) Summary of results from live imaging, showing cytoplasmic intensity relative to interphase cells (blue) and kinase assays (purple). Results from both assays are consistent.
(B-M) Representative pictures of myrLgl-GFP (B-D), Lgl-GFP (E-G), Lgl3A (H-J) and LglASA-GFP (K-M) in mitotic cells in wing disc epithelia. Note the absence of cytoplasmic localisation in myrLgl, Lgl3A and LglASA in contrast to Lgl-GFP.
(N) LglASA-GFP remains cortical during mitosis. Note the cortical localisation in a mitotic cell at the boundary of the LglASA-GFP expressing region (arrow). Scale bars 5μm.

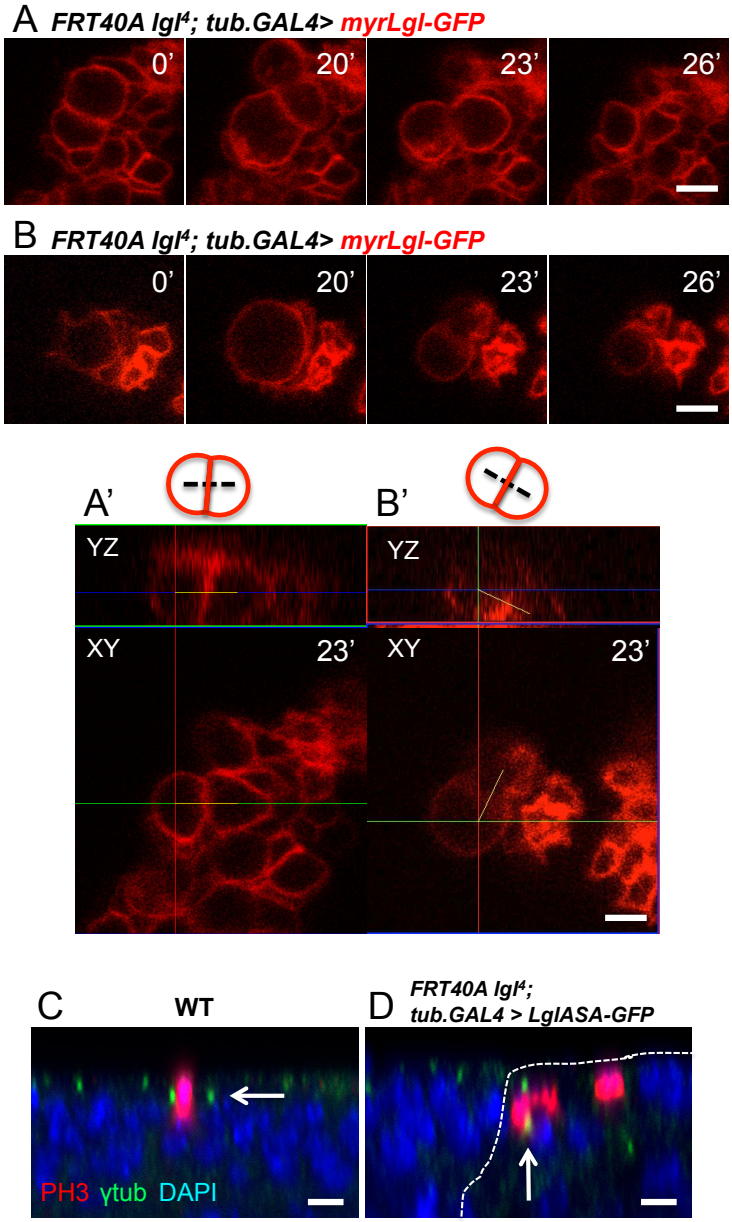

Figure A.6 Non-relocalising Lgl constructs display misorientation phenotypes in wing discs

(A-B) Still frames of *lgl*^{4} mutant clones expressing myrLgl-GFP through mitosis (from the same movie). Whilst the majority of cells divide within the plane of the epithelium (A), some cells divide away from this axis (B). YZ sections in (A') and (B') show the plane of division.
(C-D) Spindle misorientation is also seen in clones of *lgl*^{4} mutant cells expressing LglASA-GFP, as marked by γ-tub. Scale bars 5μm.

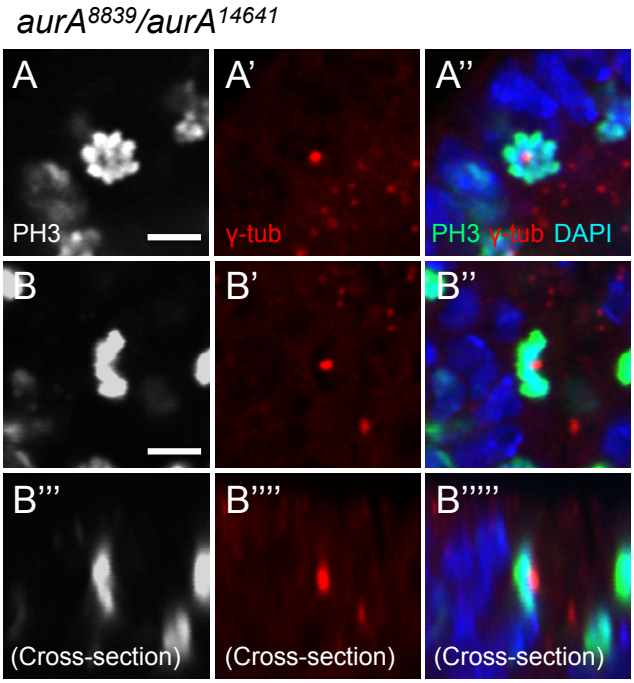

Figure A.7 Mitotic cells in *aurora A* mutant wing discs

aurora A mutant discs display an increased mitotic index, rosette structures of DNA (A), and frequent monopolar spindles (A, B), originally described in (Glover et al., 1995). These defects make analysis of spindle orientation in *aurora A* mutant discs essentially futile. Scale bars 5μm.

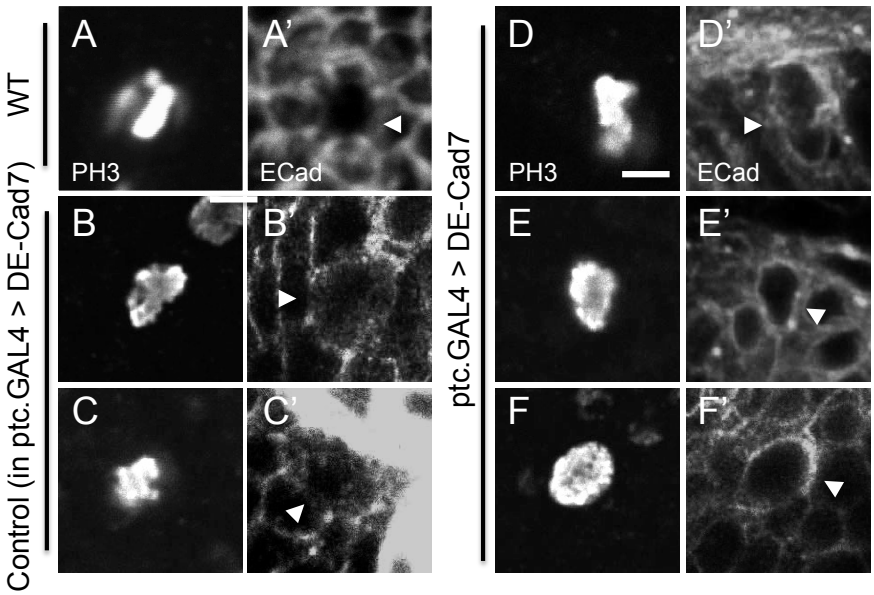

Figure A.8 Expression of E-Cadherin prevents adherens junction down regulation

(A) E-Cadherin is down regulated in mitotic cells. (B-C) In discs expressing E-Cadherin under control of ptc.GAL4, antibody staining in control cells, outside the ptc region, also reveals down regulation. (D-F) Adherens junctions in mitotic cells expressing E-Cadherin under ptc.GAL4 are no longer down regulated, as revealed by E-Cadherin staining. Numbers of mitotic cells are reduced in this region (from live imaging data). Scale bars 5μm.

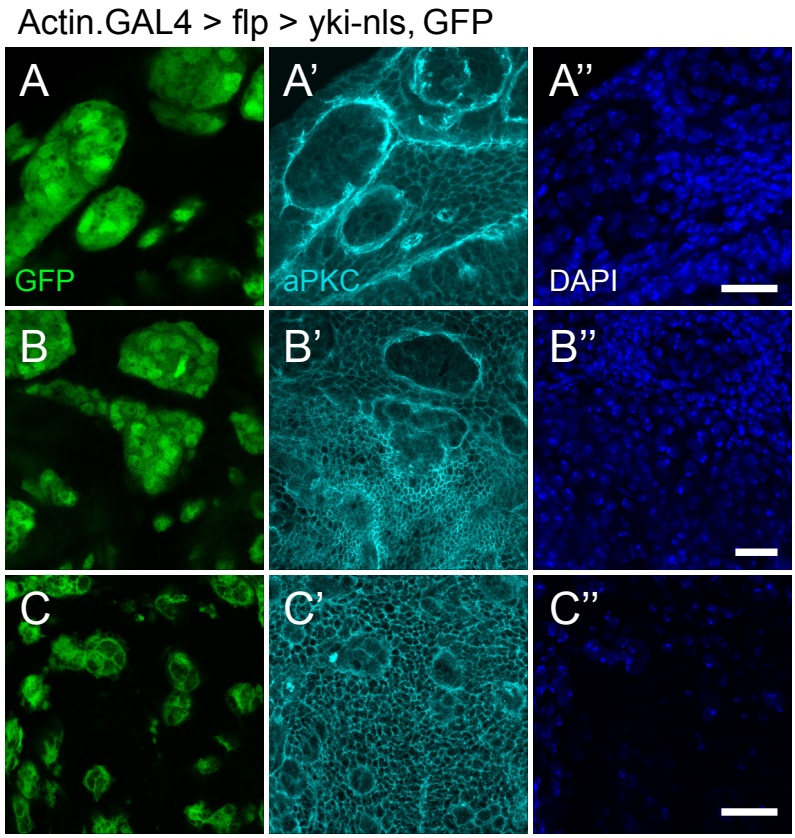

Figure A.9 Clones expressing Yki-nls form cyst-like structures with altered polarity in the wing disc

Actin flip-out clones expressing a nuclear-localised Yorkie transgene form cysts with altered polarity. Apical determinants like aPKC localise around the border of a clone, with little definition within the clone. Scale bars 20μm.

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